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THE INDUCTION OF LIVER TUMOURS BY 4-AMINOAZOBENZENE AND ITS N:N-DIMETHYL DERIVATIVE IN RATS ON A RESTRICTED DIET

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(PLATES I–VI)

THE early experiments of Yoshida (1932, 1934), Sasaki and Yoshida (1935), Kinosita (1936) and Maruya (1938), while establishing the carcinogenicity of 4'-amino-2 : 3'-azotoluene for the liver of the rat, failed to reveal any such activity on the part of 4-aminoazobenzene (AAB). Subsequent writers (Kinosita, 1939-40 ; Jacobi and Baumann, 1942 ; Kensler, Dexter and Rhoads, 1942) have stated on the basis of this early work that 4-aminoazobenzene is non-carcinogenic for the rat. In more recent experiments, Miller and Baumann (1945) fed rats for 8 months on a diet containing 0·053 per cent. of this azo-dye ; the basal diet was similar to that devised by Miller, Miner, Rusch and Baumann (1941) and found by them to favour markedly the induction of liver tumours by N : N-dimethyl-4-aminoazobenzene (DAB) administered at a molar equivalent level (0·06 per cent.) in the diet. Although the rats which had received 4-aminoazobenzene in this "pro-carcinogenic" diet for 8 months were allowed to live on the same diet, free of dye, for a further 2 months, none of them developed any liver tumours by that time, *i.e.* up to 10 months. From Miller and Baumann's experiments it is clear that the carcinogenic activity of this azo- dye, if any, is much weaker than that of its N : N-dimethyl-, or even of its N-monomethyl- derivatives, both of which induced liver tumours within 6 months in 100 per cent. of the rats given these dyes at molar equivalent levels in the same basal diet for 4 months.

In 1942, Kensler, Dexter and Rhoads reported that a diphospho-pyridine nucleotide (co-enzyme I) system was inhibited to varying degrees by free radicals of the type of Wurster's salts derived from para-diamines. They showed that the degree of inhibition by these diamines was proportional to the stability of the free radicals derived from them, estimated by Michaelis *et al.* (1939). Furthermore, they published a table in which they correlated the toxicity of some of the diamines with the carcinogenic potency towards rat liver of the azo-compound which could give rise to those particular diamines by reductive fission. According to this table, the percentage inhibitions of *p*-phenylenediamine, *p*-toluylenediamine and N : N-dimethyl-*p*-phenylenediamine were 38, 65 and 92 respectively, while the carcino-genic activities of the parent azo- dyes—4-aminoazobenzene, 4'-amino-2 : 3'-azotoluene, and N : N-dimethyl-4-aminoazobenzene respectively—increased in the same order. The fact that *p*-phenylene-diamine had an enzyme inhibition value of 38 per cent. was taken by them to indicate that azo- compounds yielding split products with an enzyme inhibition value less than a certain level (65 per cent.) would be non-carcinogenic for rat liver. This "threshold" hypothesis seems to depend on the reputed non-carcinogenicity of 4-aminoazo-benzene, whereas the toxicity of *p*-phenylenediamine might indicate that the parent azo- dye is indeed carcinogenic for rat liver, though comparatively weakly so.

Jacobi and Baumann, who demonstrated that the N-methyl groups of dimethyl-4-aminoazobenzene are labile and can take part in transmethylation, also suggested that demethylation of this azo-dye to 4-aminoazobenzene was responsible, at least in part, for the protection afforded by high-protein and high-riboflavin diets against carcinogenesis due to the dimethyl compound. This would imply that the N-methyl groups of dimethyl-4-aminoazobenzene were essential for the carcinogenic process. However, the methyl groups of the liver carcinogen, 4'-amino-2 : 3'azotoluene, are attached not to nitrogen but to carbon atoms and are not labile, which suggests that the methyl groups of dimethyl-4-aminoazobenzene may not be essential for carcinogenic activity. A prolonged test of the free base was undertaken to test this point and to assess further the theories of Kensler, Dexter and Rhoads. As soon as it was certain that AAB was carcinogenic for the rat liver a preliminary report was published (Kirby, 1944). The present paper gives full experimental data and details of the pathological findings.

<center>EXPERIMENTAL</center>

Three groups of albino rats derived from the Wistar strain were placed upon a diet designed to facilitate the induction of liver tumours by ingested azo- compounds. The "low protein" diet of Miller *et al.* (1941) was taken as a model, but war-time conditions made certain changes necessary. Thus cotton-seed oil was replaced by arachis oil, and glucose by a rather less pure source

of carbohydrate, namely potatoes. The potatoes were peeled by a machine which removed practically all the skin but not the "eyes". The composition of the diet was as follows :—

<div align="center">RESTRICTED DIET (R.D.1)</div>

Casein .	.	.	.	.	.	.	12 per cent.
Potatoes (boiled)	.	.	.	.	.	.	76 ,,
Salt (Glaxo, L.D.6)		.	.	.	.	.	4 ,,
Arachis oil	.	.	.	.	.	.	5 ,,
Cod liver oil	.	.	.	.	.	.	1 ,,
Yeast (D.C.L., dried) *	.	.	.	.	.	2 ,,

No supplements were given, other than the azo- compounds where appropriate ; the latter were added dry to freshly mixed diet each day in the amounts recorded below.

RESULTS

Group I.   Controls

Eight male and 8 female rats were maintained on the restricted diet without any azo- compound. The earliest to die, a female at 56 weeks, showed no significant abnormality of the liver, and this was true of all these control rats, including the longest lived, a male that survived for 97 weeks. The average length of life on this restricted diet was 77 weeks and would have been slightly longer if the last three survivors had not been killed. Moreover, the two weight-gain curves (fig. 1) show a considerable increase in weight in rats of either sex up to about 40 weeks, after which there was a tendency for the animals to lose weight. The males showed a greater sensitivity to the deficiencies of the diet as judged by the very rapid loss in weight between 50 and 80 weeks. The average maxima, about 300 g. for male rats and 200 g. for females, were fairly near the average maximum for normal rats of this strain maintained in our department on rat cake and only employed for breeding purposes ; in our experience these rats rarely exceed 360 g. for a male and 250 g. for a female. Miller *et al.* (1941) give no information about the health and longevity of control rats maintained on their synthetic diets ; they do, however, state that "rats on the low protein diet (0 per cent. casein) gained from 3 to 4 gm. per week in the absence of butter-yellow". This compares with gains of about 5 g. per week for male rats and about 3·5 g. per week for females during the first 14 weeks of our experiment (fig. 1). The loss in weight in our control experiment seems to have been caused mainly by the development of lung abscesses and bronchiectasis accompanied by loss of appetite. This type of lung disease is common in our strain of Wistar rats, but the deficiencies of the diet in this particular experiment seem to have exacerbated the lesions. One

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* The makers of this dried yeast informed us that the riboflavin content was 1·4 units per oz. Thus 100 g. of the diet R.D.1 would contain approximately 0·1 mg. A rat consuming 12·5 g. of this diet per day would therefore receive 12·5 µg. daily ; this amount of vitamin is not above the 15 µg. stated by Kensler *et al.* (1941) to be needed for normal health in the rat and well below the 200 µg. found to be protective (in conjunction with a high casein level) against carcinogenesis by N : N-dimethyl-4-aminoazobenzene.

type of lung lesion, concomitant with and probably related to the infected state of the lungs, was a change to flattened or low cubical
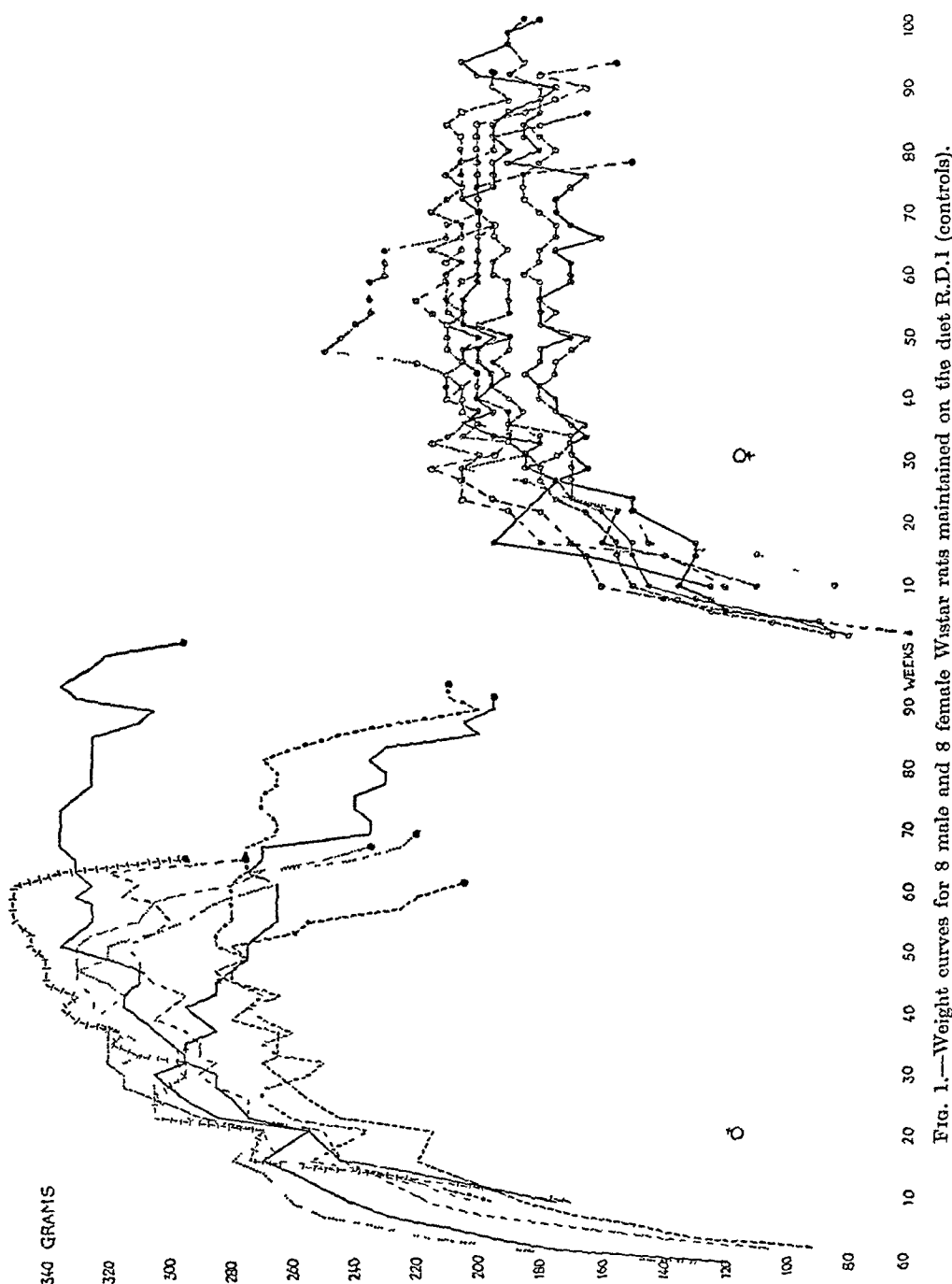
FIG. 1.—Weight curves for 8 male and 8 female Wistar rats maintained on the diet R.D.1 (controls).

epithelium of the terminal bronchioles, producing, on occasion, an appearance of proliferated ducts not unlike that seen in a liver which

is the seat of bile-duct hyperplasia. Fig. 4, from control rat 208, shows the origin of these unusual structures. The importance of recognising the non-neoplastic nature of this epithelial modification, found in the seven control rats of which the lungs were examined microscopically, will be apparent from the results recorded later in this paper. No livers in this group showed proliferative changes and only a few showed degenerative changes.

Group II. N : N-dimethyl-4-aminoazobenzene

(a) *Diet R.D.1.* As neither the basal diet nor the strain of rats could be regarded as strictly comparable with those used by Miller *et al.* (1941), it was considered necessary to investigate the action of
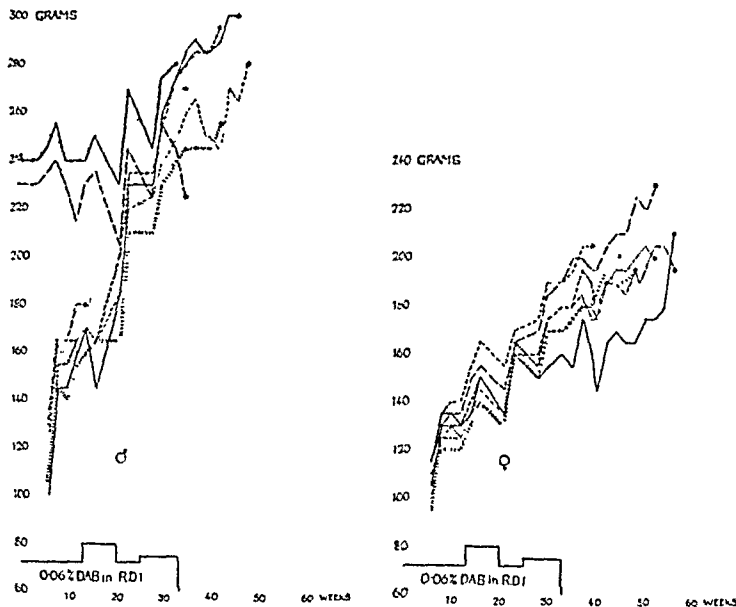
Fig. 2.—Weight curves for 8 male and 7 female Wistar rats maintained on the diet R.D.1 with supplements of N : N-dimethyl-4-aminoazobenzene, at levels indicated, for 33 weeks.

the well established carcinogen N : N-dimethyl-4-aminoazobenzene (DAB) under our conditions. Eight male and 7 female rats were therefore given the basal diet with this azo- dye added. The initial level was 0·06 g. per 100 g. of diet. An attempt was made to force up the level of the dye to 0·1 g. per 100 g. of diet, but the animals began to lose weight (fig. 2) and the level was reduced to 0·06 g.

again, although the later stages were carried out successfully with a diet containing 0·07 g. of dye per 100 g. of diet. After 28 weeks 4 male rats in one cage were taken off the dye and given the diet only, to observe the effect, if any, upon the degree of cirrhosis persisting at eventual death ; the remaining rats were taken off the dye at 33 weeks.

Rats in this group were killed when liver tumours were definitely palpable. One rat was eaten by cage mates after 7 weeks. Apart from this, the first rat died after 29 weeks with large tumours in the liver. Histological examination revealed adenocarcinoma and hepatoma as well as cirrhosis. All the remaining rats were found at autopsy to have liver tumours of one kind or another. One had metastases of a hepatoma in the mesentery. The average survival period was 40 weeks from the beginning of dye feeding, but tumours were usually palpable 4-10 weeks before death and the average time of tumour induction would have been 33 weeks or less. Four female rats which survived 14 weeks or more after dye feeding had ceased showed little or no cirrhosis, but the livers contained tumours definitely malignant histologically.

In the strain of albino rats used and with the restricted diet employed, DAB given orally at a level of 60-75 mg. per 100 g. of diet induced liver tumours of one kind or another in all animals that survived for 10 months or more. It might be concluded that the diet R.D.1 protected the rats as compared with the diet used by Miller *et al.* (1941, 1945), whose rats all developed liver tumours by 6 months.

(b) *Diet R.D.*3. It has since been possible to carry out another experiment with the same dye, at a level of 0·07 per cent., in rats of the same Wistar strain, but using a diet—R.D.3—in which potatoes were replaced by starch. This allows of more accurate comparison with the results of the American workers, who used glucose as carbohydrate. The weight curves are shown in fig. 3. Contrary to expectation, liver tumours developed even more slowly on this new diet ; even in 6 rats which survived one year, 4 had no gross liver tumours at autopsy. Out of 16 rats used, only 3 showed definitely malignant liver tumours. It seems, therefore, that the slower rate of development of tumours due to DAB in our experiments compared with that in the experiments of Miller *et al.* (1941) is related to the strain of rats used rather than to the differences in diet (unless there is some protective factor in starch as compared with glucose). Apparently Sprague-Dawley rats are much more susceptible to carcinogenesis of the liver by DAB than are Wistar rats.

Nevertheless the lesions found in rats given DAB, especially frequent on the diet R.D.1, were *essentially the same as those* reported by other workers after feeding rats with this dye. Bile ducts frequently underwent cystic dilatation, so that large clear cysts sometimes appeared on the surface of the liver. Bile-duct proliferation, with or without cirrhosis and cholangiofibrosis, was also common on

both diets, and in 8 cases out of 27 adenocarcinoma was found. Twelve out of 15 rats receiving DAB in R.D.1 developed hepatomas which were considered in 8 cases to be malignant, but on R.D.3 + DAB the rats showed hepatomas in only 4/12 cases and only one of these appeared to be malignant. Thus bile-duct neoplasia was as frequent as liver-cell neoplasia in rats given R.D.1 + DAB, and considerably more frequent in rats given DAB in R.D.3. This tendency for
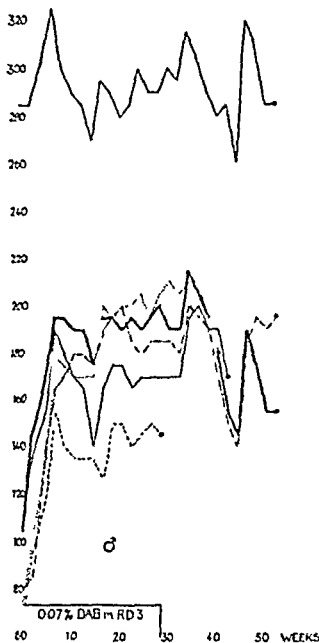
Fig. 3.—Weight curves for 6 male and 6 female Wistar rats maintained on the diet R.D.3 with supplements of $N : N$-dimethyl-4-aminoazobenzene, at levels indicated, for 29 weeks.

tumours derived from proliferating bile ducts to predominate agrees with Orr's findings (1940). Edwards and White (1941-42) have differentiated more than one type of hepatoma in their material; they have shown by transplantation into homologous rats that their hepatoma type I is benign, and that type II is malignant. In our experience, more than one histological type of neoplasia was often present in a single liver and even in individual tumour nodules. In many instances sections of liver would show cirrhosis, cholangiofibrosis,

cholangioma, adenocarcinoma and hepatoma within an area of a few square mm. Where multiple tumours are present in the liver it is not safe to assume that all have the same histogenesis, and unless many portions of tissue are examined in such cases, the histological classification of the tumours has little value. The great variety of neoplastic changes to be seen in an area of only 2-3 mm. diameter is illustrated in fig. 5, which was prepared from one section of a tumour in rat 190 (given DAB in R.D.1 for 262 days—total dye 2·37 g. approx.); figs. 6 and 7 show larger magnifications of areas in the same liver.

Orr (1940, p. 404) states that cystadenomas in his material often resembled "the common cavernous hæmangioma of the human liver", particularly when the lining cells were of flattened "endothelial" type, but they rarely contained any blood. Such essentially benign cystadenomas often occurred in our material, but hyperplasia of sinusoids was seen in several cases and in some of these the blood vessels seemed to have become neoplastic, growing without obvious relation to the needs of the tumour and even apart from the hepatoma. In some areas it was impossible to distinguish between endothelial and epithelial cells, both types apparently being involved in the malignant process, so that sinusoids with defective walls opened directly into epithelial structures filled with blood in which many detached malignant cells could be seen in sections (figs. 8-10).

Rats in these sub-groups also developed lung lesions. Bronchiectasis was only found in one rat of each sub-group, but of the rats given DAB in the diet R.D.1 several showed flattening of the epithelium of the terminal bronchioles. When septic areas of lung contained such altered bronchioles the sections had an appearance suggesting secondary deposits of adenocarcinoma, an interpretation especially attractive as so many rats in this group had cholangiomas of the liver. However, the limited extent of such structures and their similarity to the lesions seen in the control rats make it reasonably certain that they were primary and non-malignant, even non-neoplastic. To illustrate the histological similarity of the lung and liver lesions there are shown, in figs. 11 and 12, a section of liver and one of lung from rat 190 (262 days on DAB in R.D.1).

The "pro-carcinogenic" influence of the restricted diets used by us calls for further comment. The much greater and earlier incidence of liver tumours obtained by Miller et al. (1941, 1945) in Sprague-Dawley rats given DAB orally compared with that found by us in Wistar rats receiving the same dye at the same dietary level is considered to be due to differences in strain of animals rather than to differences in diet. Our diet R.D.3 differed from that designed by Miller et al. (1941) in the replacement of cerelose (a pure, commercial glucose monohydrate) by starch, of cotton-seed oil by arachis oil, and of Vitab (a commercial rice-bran extract) by dried baking yeast. In their "rice-carrot" model diet, Miller et al. (1941) also used yeast

EXPERIMENTAL LIVER TUMOURS

Fig. 4 Rat 208, control. Early bronchiectasis. Note similarity of terminal bronchioles to proliferated bile ducts (see fig. 11). H. and E. × 280

Fig. 5.—Rat 190. Fed with N : N-dimethyl-4-aminoazobenzene 230 days. Killed 263rd day. Trabecular hepatoma and adeno-carcinoma, apparently derived from proliferated bile ducts. Cirrhotic bands traverse the field. H. and E. × 90.
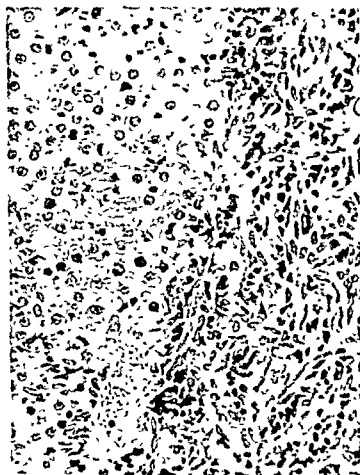
Fig. 6.—Rat 190. Another field showing junction between nodular hyperplasia and undifferentiated connective tissue from area of incipient cirrhosis. H. and E. × 280.

Fig. 7.—Rat 190. Cholangiofibrosis showing bile ducts distended with mucus. H. and E. × 280.

PLATE II

EXPERIMENTAL LIVER TUMOURS
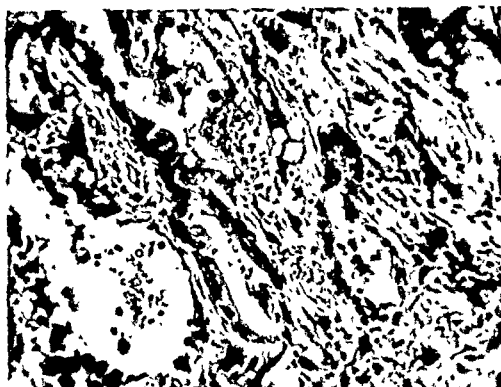
FIG. 8.—Rat 197. N : N-dimethyl-4-aminoazobenzene 230 days. Killed 350th day. Mixture of adenocarcinoma and hemangioma in liver. H. and E. × 280.
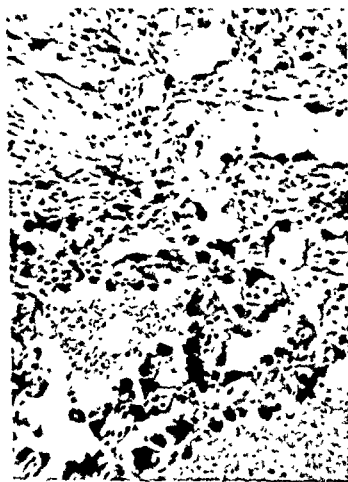
FIG. 9.—Rat 197. Area showing *endothelial* cells budding off into dilated sinusoids of liver. H. and E. × 280.
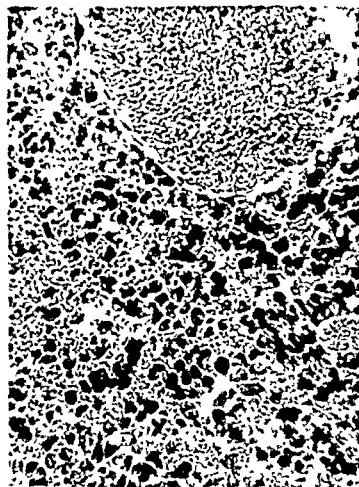
FIG. 10.—Rat 202. N : N-dimethyl-4-aminoazobenzene 230 days. Died 254th day. Metastasis in mesenteric lymph node showing undifferentiated malignant cells apparently of endothelial origin. Walls of sinusoids defective except in upper part of field. H. and E. × 280.

PLATE III

EXPERIMENTAL LIVER TUMOURS
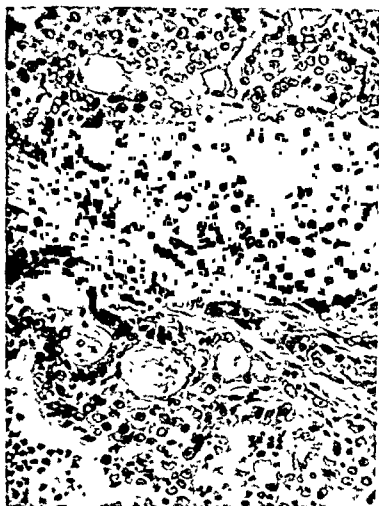
Fig. 11 —Rat 190   Irregular bile duct hyperplasia and adenocarcinoma secondarily infected   H. and E   ×280
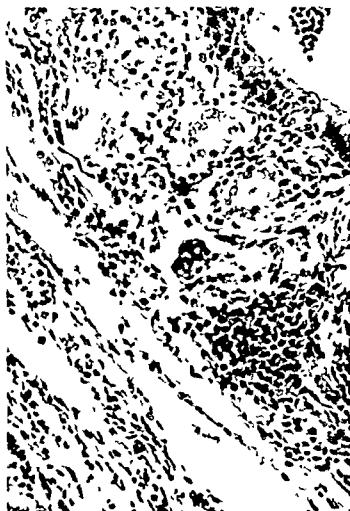
Fig. 12 —Rat 190.   Abnormal terminal bronchioles showing cubical epithelium, for comparison with fig 11.   H. and E.   ×280.

Fig 13 —Rat 139   4-Aminoazobenzene 527 days   Multiple tumours in left ventral lobe of liver.

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Experimental liver tumours

Fig. 15.—Rat 138. Permeation of vein in Glisson's capsule by malignant hepatoma. Note similarity of appearance between cholangiofibrotic ducts in lower half of field and abnormal terminal bronchioles in cases of early bronchiectasis (figs. 4, 12, 22 and 23). H. and E. × 280.
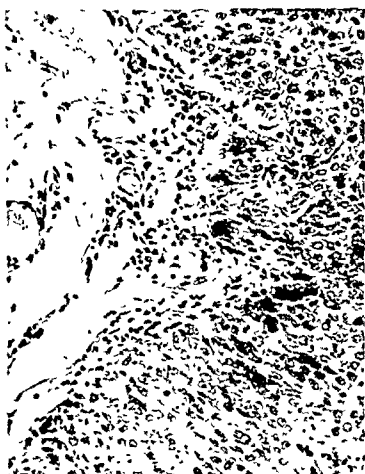
Fig. 16.—Rat 139. Metastasis in mesentery from malignant hepatoma. H. and E. × 240.
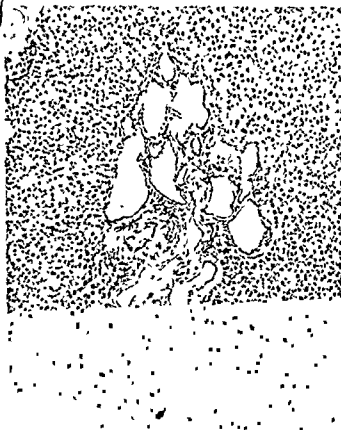
Fig. 17.—Rat 139. Cholangiectasis due to pressure by tumours. H. and E. × 90.

Fig. 18.—Rat 138. Cystadenoma and fine cirrhosis. H. and E. × 170.

EXPERIMENTAL LIVER TUMOURS
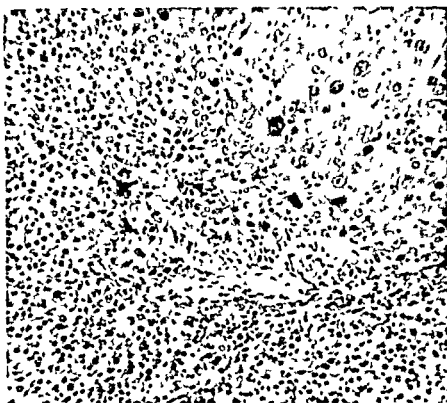
FIG 19—Rat 138   4-Aminoazobenzene 525 days   Nodular hepatoma composed
of very large cells   H. and E   ×170
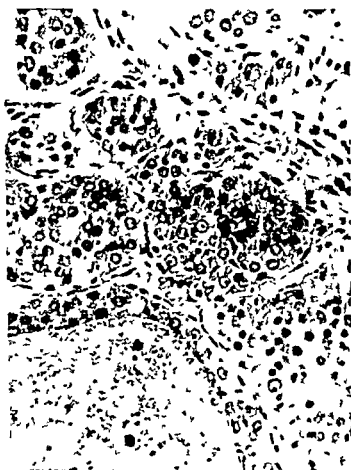
FIG 20—Rat 133   4 Aminoazobenzene 640
days   Hæmangiohepatoma   H. and E.
×280

FIG 21—Rat 138   Cholangiofibrosis and fine
cirrhosis   H and E   ×170

PLATE VI

EXPERIMENTAL LIVER TUMOURS
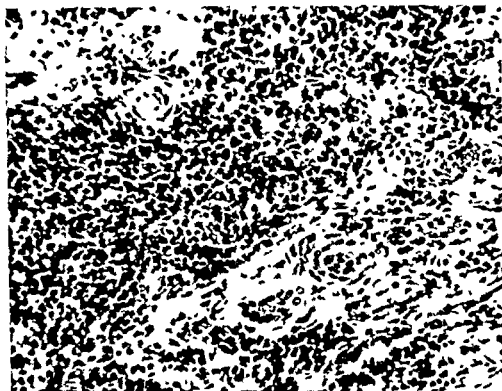
FIG 22.—Rat 143   4 Aminoazobenzene 702 days   Heavily infected lung.   Small bronchioles with flattened epithelium.   H. and E   ×280.

FIG 23.—Rat 136   4 Aminoazobenzene 673 days and then killed   Lung showing early bronchiectasis   Note flattened epithelium of terminal bronchioles, some of which contain pus   H. and E   ×280

instead of Vitab, without significant effect on their results. Miller *et al.* (1944) have shown that replacement of corn oil by hydrogenated cotton-seed oil in the diet containing DAB results in a greatly reduced incidence of liver tumours, but arachis oil and cotton-seed oil resemble corn oil closely in chemical constitution and there is good reason to expect it to yield similar results. On the other hand, our diet R.D.1 replaced cerelose by boiled potatoes, which not only contain substances other than carbohydrates but also contain about 75 per cent. water. Thus while an animal ingesting the same weight of R.D.1 as of R.D.3 would receive similar weights of protein, salts, oil and yeast, it would receive considerably less carbohydrate, and the ratio of proximate principles would be quite different. For comparison, the amounts of the various constituents for R.D.3 and R.D.1 are set out in table I. It will be seen from the figures in the last column that a

TABLE I

Percentage composition of the restricted diets

	R.D.3	R.D.1	
	Per cent.	Per cent.	Ratio
Casein	12	12	48
Starch	76	0	0
Potato (dry material) . . .	0	19 } 76	76
Water	0	57 } 76	0
Salts	4	4	16
Arachis oil	5 } 6	5 } 6	24
Cod liver oil	1	1	
Dried yeast	2	2	8
	100	100	...

rat eating enough R.D.1 to get the same amount of carbohydrate as a rat eating R.D.3 would ingest four times as much protein (casein), salts, oil and yeast, and therefore riboflavin. It would thus appear that rats receiving sufficient R.D.1 were not deficient in protein or riboflavin. This presumably explains the reasonably good growth seen in all rats receiving R.D.1, although the controls on R.D.1 alone never reached the weights attained in similar rats given a satisfactory balanced diet, namely rat cake (Thomson, 1936). Rats maintained on R.D.1 with or without DAB grew faster and reached heavier weights than rats on R.D.3 + DAB ; they also showed a higher incidence of tumours. The reason for these results is not clear.

The weight of each diet consumed per rat was about the same in each group, but the caloric value of R.D.1 was lower, owing to the 57 per cent. of water in this diet. On the other hand, the food intake of the R.D.1 group must have been at least as adequate as that of the R.D.3 group, judged by their body weights. The higher ratio of

protein to carbohydrate in R.D.1 may account for the greater rate of growth but does not explain the higher incidence of liver tumours.

Whatever the explanation, the fact remains that a more normal diet—R.D.1—containing more " protective " substances, favoured carcinogenesis more than a " less normal " diet—R.D.3—containing less of these substances.

Group III. 4-Aminoazobenzene

Sixteen male rats were used in this experiment. The basal diet (R.D.1) was the same as for groups I and II(a). The dye was added
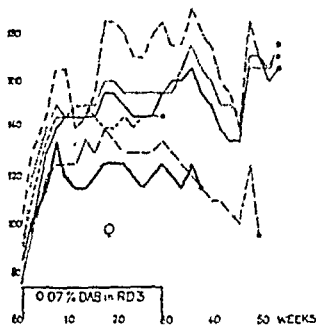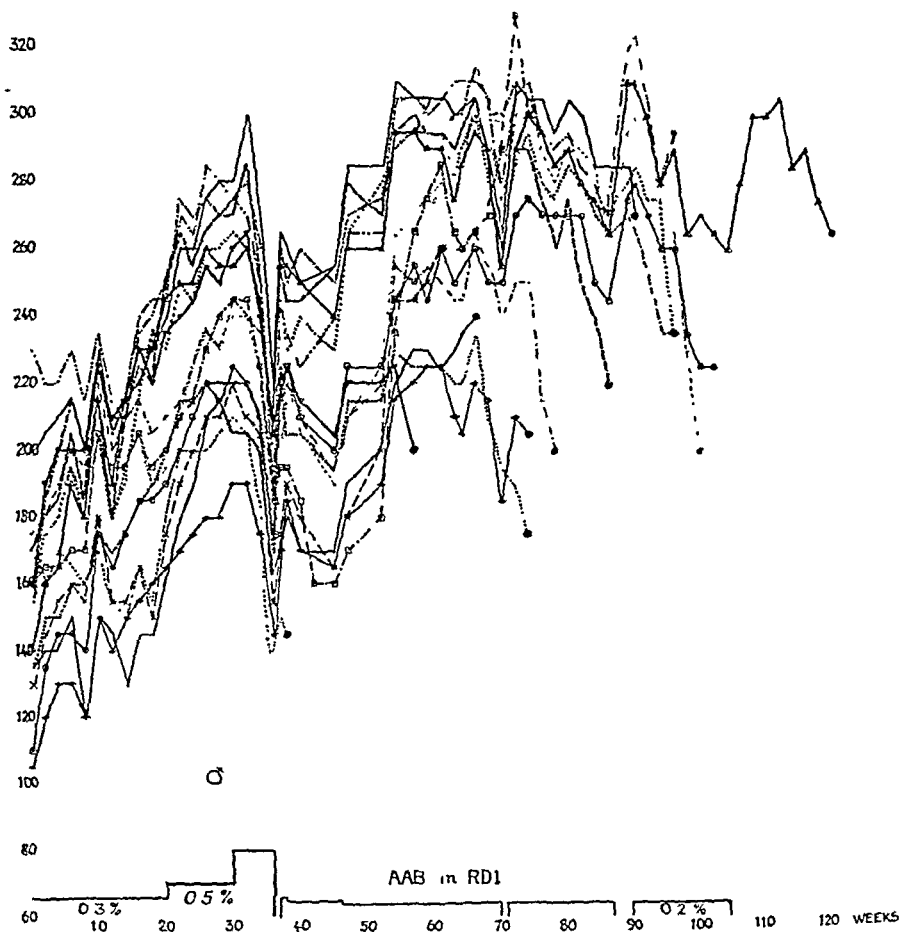

FIG. 14.—Weight curves for 16 male Wistar rats maintained on the diet R.D.1 with supplements of 4-aminoazobenzene, at levels indicated, up to 2 years.

initially at the level of 0·3 per cent., and later, after 20 weeks, at the level of 0·5 per cent. of the diet. These levels are roughly 5 and 8 times respectively the level at which the dimethyl derivative can

safely be given, but the weight curve (fig. 14) indicates that a reasonable increase in weight was maintained up to the 26th week. At the 30th week the dye level was raised to 1·0 per cent. and this was soon followed by a sharp fall in weight, ending at the 36th week, when the dye was withdrawn for one week. At the 37th week the dye was again added to the diet at a level of 0·3 per cent., but the rat weights (which had largely recovered in the absence of the dye) began to fall again, and the dye level was reduced to 0·25 per cent. and, at the 45th week, to 0·20 per cent., when a satisfactory rise in the weight curves set in. That the presence of the dye was retarding growth is shown by the sharp temporary rises at 71 and 87 weeks respectively, when the dye was withdrawn for a short time. One of the two survivors at 104 weeks was killed; the other was allowed to live on to 120 weeks on the restricted diet but without any further dye. As will be seen from fig. 14, this rat at first increased in weight after the dye was finally withdrawn, an improvement which was not maintained.

Smith *et al.* (1943) found that Wistar rats tolerated 4-aminoazobenzene at a level of 0·1 per cent. in a low protein diet, but the weights of the animals fell by as much as 20 per cent. in the course of 5 weeks. When the same amount of dye was incorporated in a diet containing 27 per cent. of casein, 8/11 rats gained weight up to 50 per cent. in 6 weeks. These were essentially short-term experiments and provide a different kind of evidence from that reported in this paper. However, it is clear that 4-aminoazobenzene is a toxic substance for the rat but that it may be tolerated fairly well up to a level of 0·2 per cent. of the diet, especially if that diet is rich in first-class protein.

Contrary to the findings of previous workers, we found that AAB could induce malignant liver tumours in a proportion of rats (table II). But the earliest neoplastic lesion was found in a male rat killed at 400 days, and malignant tumours were not found until 525 days had passed. The latter period exceeds that used by any previous workers; moreover the diet contained a higher proportion of the dye. However, tumours of undoubted malignancy appeared in only 2 out of the 16 rats used; one of these two livers, which weighed 47·8 g., is illustrated in fig. 13. The tumours were primary liver-cell carcinomas. One showed invasion of the veins of the liver capsule (fig. 15), the other metastasis to the mesentery (fig. 16). While bile-duct proliferation and cystadenomas (figs. 17 and 18) were found in these two livers and in the livers of 3 other rats surviving longer periods, no adenocarcinomas were found in the liver of any rat of this group. It seems that methylation favours the induction of malignant tumours derived from bile ducts as judged by the results obtained with DAB. It would appear, moreover, that methylation of the amino- group is much more effective than methylation of carbon atoms in the 4-aminoazobenzene molecule. Emmart (1940-41)

reports benign and malignant hepatomas in Wistar rats given 4'-amino-2 : 3'-azotoluene, the only reference to bile-duct hypertrophy being made in a histological report on one rat. Miura (1935), summing up the results of Sasaki's group in Tokyo, states that only 4 typical cholangiomas were found in white rats given this dye, whereas many hepatomas were found in rats fed longer than 135 days. Miller and Baumann report greater carcinogenic activity for the liver of rats

TABLE II

Incidence of certain lesions in relation to dye, diet and sex

Group	Sex	Stomach	Kidney		Liver					Spleen	Lung
		Papilloma-tosis	Pigmenta-tion of tubular epi-thelium	Necrosis of con-voluted tubules	Bile duct prolifera-tion	Bile duct tumours	Hepa-toma	Liver cell carci-noma	Cirr-hosis	Enlarge-ment	Abscesses (bronchi-ectasis)
Controls on R.D.1	M	0/8	0/8	1/8	0/8	0/8	0/8	0/8	0/8	0/8	5/8
	F	1/8	0/8	3/8	0/8	0/8	0/8	0/8	0/8	0/8	5/8
DAB in R.D.1	M	0/8	1/8	0/8	6/8	5/8 4 M *	7/8	5/8	6/8	1/8	1/8
	F	0/7	0/7	0/7	6/8	4/8 2 M *	5/7	3/7 1 S †	4/7	2/7	0/7
DAB in R.D.3	M	0/6	0/6	4/6	4/6	6/6 1 M *	2/6	0/6	1/6	0/6	0/6
	F	0/6	0/6	2/6	3/6	4/6 1 M*	2/6	1/6	3/6	2/6	1/6
AAB in R.D.1	M	6/16	10/16	2/16	6/16	0/16	5/16	2/16 2 S †	6/16	15/16	12/16

* M = malignant.　　　　† S = metastases.

(presumably Sprague-Dawley) on the part of m'-methyl-N : N-dimethyl-4-aminoazobenzene than that possessed by DAB, but unfortunately make no mention of the type or types of lesion induced. These findings seem to indicate that the extra carcinogenic activity imparted by the N-methyl groups is due to an effect on bile ducts ; the further increase imparted by methylation of the m'-carbon atom is probably due to an induced greater lability of the N-methyl groups, which is actually decreased when methyl groups are introduced on other carbon atoms of the DAB molecule.

Hepatomas were found in 7 rats all told, which died or were killed at 400, 525, 527, 548, 620, 702 and 730 days respectively ; cystadenomas of bile ducts but no hepatomas were found in the last rat, killed at 841 days. Various types and stages of hepatomas are illustrated in figs. 19 and 20, including hepatoma in association with angioma. The types of lesion were essentially similar to those obtained with DAB, except that hepatoma predominated over bile-duct neoplasia whereas the reverse was true for rats receiving DAB.

Some degree of cirrhosis (fig. 21) was found in 6/16 rats in the AAB group but it was less severe than in the DAB group. This may

account for the higher incidence of cholangioma in the latter group, as bile duct proliferation regularly accompanies the development of cirrhosis and may lead either to the essentially benign and ultimately atrophic lesion first described by Edwards and White and termed by Opie cholangiofibrosis, or to cholangioma which according to Opie (1944) originates as a rule from areas of cholangiofibrosis.

Splenic enlargement up to 10 times normal weight was a regular feature of the AAB-fed rats and 5 autopsy specimens weighed 5·75, 6·0, 6·35, 6·75 and 7·3 g. respectively. Such gross enlargement cannot be accounted for by the degree of cirrhosis of the liver and accompanying back pressure, as the DAB-fed rats showed greater evidence of cirrhosis with little splenic enlargement. Spleens from the AAB group contained great numbers of ·   ·   ·   ·   · · · · cells in the pulp, most of which gave th   ·   ﹨ all the pigmentation was due to hæmosiderosis, however, and yellow iron-free pigment was present similar to that seen in the renal convoluted tubules of the same animals. It is suggested that enlargement of the spleen in the AAB group is initially due to a toxic effect on the red corpuscles and is part of a compensatory mechanism.

Pigmentation of the epithelium of the convoluted tubules of the kidney was reported in DAB-fed rats by Edwards and White and in rats given azobenzene, AAB or DAB by Smith *et al.* Pigment was absent or very slight in our DAB-fed rats on either basal diet, but in 10 of the AAB-fed rats the convoluted tubules were loaded with brown pigment which, like that seen by Edwards and White in DAB-fed rats, did not give the Prussian blue reaction. In our experiments AAB was given in much larger quantities than was DAB, and this may account for these differences. In control rats there was no pigmentation in the kidneys, which were not always free from damage, however. In 6 controls the kidneys showed some evidence of toxic change, 4 showing necrosis of the convoluted tubules. Essentially similar renal lesions were seen in 2 male rats given DAB in diet R.D.1 and also in a few rats given DAB in diet R.D.3 and in 6/16 rats given AAB in diet R.D.1. It seems, therefore, unlikely that the azo- dyes were responsible for kidney changes other than pigmentation.

Although 12/16 rats in the AAB-fed group developed bronchiectasis compared with 1/15 and 1/12 in the DAB-fed groups, the control rats developed similar lesions in 10/16 cases, and it seems clear that the DAB-fed rats were only freer from this lesion because they died or were killed at a much earlier age. A number of rats in the AAB-fed group showed flattening of the epithelium in the terminal bronchioles, which when associated with gross bacterial infection gave rise to the curious histological appearances already described and which might be mistaken for metastatic adenocarcinoma (fig. 22). Serial sections of these lungs revealed continuity between the bronchioles with flattened epithelium and larger bronchioles or bronchiectatic cavities

(fig. 23). Moreover, the observance of similar epithelial changes in control animals (fig. 4) showed that the dye could not be held either directly or indirectly responsible.

Edwards and White report 4 cases of papilloma of the forestomach in their DAB-fed rats. Smith *et al.* make no reference to stomach lesions in rats given DAB, either on high- or low-protein diets, but they state that papillary keratosis was seen in rats receiving azobenzene or AAB in low-protein diets, and that this lesion of the forestomach " is commonly seen in low-protein, yeast-containing diets regardless of the presence or absence of toxic substances in the diet ". While none of our DAB-fed rats showed this type of lesion, one female rat receiving R.D.1 without any azo- dye had only this papillary keratosis, and 6/16 rats receiving AAB in R.D.1 showed naked-eye ulcero-papillomas in the forestomach, usually close to the margin of the glandular zone. These lesions may be indirectly related to the action of the azo- compounds, which possibly induce an avitaminosis A such as occurs in rats fed on overheated fats (Beck and Peacock, 1941).

CLASSIFICATION OF INDUCED HEPATIC TUMOURS

The remarkable regenerative capacity of the liver renders difficult the classification of tumours of this organ. Various terms are used by different pathologists to describe a range of neoplastic growths ranging from benign cholangioma to benign hepatoma, with less easily distinguished malignant tumours bridging the histological gap between these extremes. Opie has reviewed this aspect of the pathogenesis of liver tumours induced by DAB. His classification and the conclusions he draws from his own and others' work are applicable to most of the tumours in our experiments with DAB and AAB. There is however a type of trabecular hepatoma associated with many dilated sinusoids which may be termed hæmangiohepatoma. The vascular component of these tumours seems to exceed the limits of a stroma reaction and has the appearance of a cavernous angioma. Such tumours are distinct from those cystic tumours into which hæmorrhage has occurred and in which no endothelial lining to the blood spaces is present. The pleomorphism of induced liver tumours has been noted by most experimental pathologists. In the present series of experiments many livers show the utmost confusion of histological types in some regions, though well differentiated multiple tumours are also present. It seems clear that a single carcinogen of the azo- group may give rise to multiple tumours, sometimes of several histologically distinct types, and that these may invade each other and thus produce a confused pattern. Metaplasia occurring in hepatomas or in cholangiomas may also account for some of the pleomorphism of the final tumour.

Sometimes the origin of hepatic adenocarcinoma can be traced directly to groups of hyperplastic bile-ducts, but apparently identical

adenocarcinoma seems also to arise from trabecular hepatomas. Decision as to malignancy is often difficult in the absence of metastasis. In human pathology, well differentiated hepatomas in which the cells contain bile pigment inclusions sometimes give rise to metastases of similar histology. Thus it seems unjustifiable to base estimates of malignancy on cytological appearances alone. In experimental hepatomas the gradation from benign to anaplastic malignant types includes stages where it is impossible to say whether the tumour is simple or malignant. From the practical standpoint, however, any substance that induces neoplasia of any epithelial component of the liver should be classed as a carcinogen.

OTHER CONSIDERATIONS

It is curious that no sarcomas have occurred in this series, although our Wistar rats occasionally develop sarcoma in the fibrous capsule around encysted *Cysticercus fasciolaris.* One might have supposed that the proliferating connective tissue in areas of early cirrhosis would be liable to develop sarcomas in these animals, the more so as azo- compounds have been shown to be sarcogenic for the connective tissues of mice.

No doubt with increasing knowledge it will be possible to classify chemical carcinogens more precisely into epitheliotropic and meso-dermotropic groups, but it would seem that local metabolic differences may account for the variable response of the connective tissues in different sites to particular carcinogens. It is also certain that rats and mice differ in their susceptibility to many carcinogens.

The results obtained by Glynn et al. (1945) showing the role of methionine and cystine in preventing liver necrosis and their differentiation between necrosis of dietary origin and cirrhosis offer better prospects of experimentally controlled carcinogenesis un-complicated by histologically confusing reactions. It would appear from their work that 7 per cent. of casein is near the minimum supply of sulphur-amino-acids to prevent massive necrosis of the liver. Although up to 1·0 g. of 4-aminoazobenzene per 100 g. of the diet R.D.1 (which contained only 12 per cent. of casein, wet weight) was given during the first 35 weeks of our experiment, no massive necrosis or scarring was seen in any of the rats dying at any time, including the first which died in the 39th week. Moreover, retardation of growth was not serious and actual loss of weight began even later than in the control group (figs. 2 and 14). In the experiments of Glynn et al., rats on diets deficient in methionine began to lose weight at once. It would therefore appear that even large doses of 4-aminoazo-benzene, when the intake of sulphur-amino-acids is relatively low, do not seriously interfere with the supply of sulphur for normal metabolism.

<center>CONCLUSIONS</center>

The conclusion may be drawn that 4-aminoazobenzene (AAB) is a carcinogen of which the activity is similar to, though much weaker than, its N : N-dimethyl derivative (DAB). It causes less cirrhosis and less bile-duct hyperplasia than does DAB and the neoplastic changes induced by AAB affect liver parenchyma rather than bile-ducts, whereas DAB induces tumours of both types of cell.

While belief in the essential character of the methyl groups in DAB is somewhat reduced by our findings with AAB, the peculiar importance of these groups is obvious. Moreover, it has been emphasised by the recent observations of Sugiura *et al.* (1945) that higher homologues of DAB are quite inactive in the rat. Apparently the liver of the rat is able to methylate or demethylate, but the corresponding processes with ethyl (or higher) groups cannot be carried out. This accords with the results observed with ethionine in rats by Dyer (1938), and it seems likely that N : N-diethyl-4-amino-azobenzene is non-carcinogenic for the rat liver because that organ is unable to de-ethylate it. Reductive fission should yield N : N-diethyl-*p*-phenylenediamine and this would probably be excreted as the N'-acetyl derivative. Unfortunately, the experiments carried out by Kensler, Dexter and Rhoads (1942), Kensler, Young and Rhoads (1942), Potter (1942) and Elson and Hoch-Ligeti (1944) did not include the effect of N-ethyl-*p*-diamines on enzyme systems, and the inhibitory action, if any, of N'-acetyl-N : N-diethyl-*p*-phenylenediamine cannot be related to the non-carcinogenicity of the parent azo- dye.

The experiments of Miller, Miller and Baumann (1945) on the methylation and demethylation *in vivo* of azo- dyes may not be valid for Wistar rats under our experimental conditions. Hence it cannot be decided without further experiment whether 4-aminoazobenzene is carcinogenic *per se* or acts by virtue of conversion in the liver to the dimethyl derivative.

It is noteworthy that attempts to induce liver tumours in stock mice by repeated subcutaneous injections of AAB dissolved in arachis oil failed completely, even after 626 days' experimentation and a total dose of 192·5 mg. Since, however, the dimethyl compound DAB is so very weak a carcinogen for mice, whether administered by feeding or by subcutaneous injection (Law, 1941 ; Andervont and Edwards, 1942-43 ; Andervont, White and Edwards, 1943-44 ; Kirby, 1945*a*), it is not perhaps surprising in view of the results in rats that AAB should be non-carcinogenic in mice ; yet methylation of certain carbon atoms to yield 4'-amino-2 : 3'-azotoluene produces a very potent hepatic carcinogen for mice (for references see Kirby, 1945*b*). This reversal of the carcinogenic potencies in rats compared with mice of DAB and 4'-amino-2 : 3'-azotoluene and its bearing on theories of azo- compound carcinogenesis have already been discussed (Kirby, 1945*b*).

SUMMARY

1. 4-Aminoazobenzene has been shown to be carcinogenic for the liver of the Wistar rat when given orally at a level of 0·2 per cent. or more in a diet, R.D.1, favourable to the production of liver tumours by azo- dyes in this strain of rat.

2. The carcinogenic action of this azo- dye was found to be much less than that of its N : N-dimethyl derivative, which was given at a lower level in the same basal diet, R.D.1, to rats of the same strain.

3. The basal diet used, R.D.1, was found to be more favourable to liver-tumour induction by N : N-dimethyl-4-aminoazobenzene than was another basal diet, R.D.3, which approximated more closely to that devised by Miller *et al.* (1941) and found by them to favour greatly liver-tumour induction by this azo- dye in Sprague-Dawley rats.

4. 4-Aminoazobenzene caused little cirrhosis and few tumours of bile-duct origin ; the N : N-dimethyl derivative caused considerable cirrhosis, and bile-duct tumours exceeded those of liver-cell origin. Rats given the free base also developed greatly enlarged spleens ; in those given the methylated base the spleen was not significantly enlarged.

5. Primary tumours were not found in organs other than the liver. Metastases were found from liver tumours due to 4-aminoazobenzene as well as from those due to the dimethyl derivative.

6. The presence of methyl groups on the amino group of 4-amino-azobenzene does not appear to be essential for carcinogenesis, but this action is greatly enhanced by such methylation.

7. The classification of induced hepatic tumours is discussed and their diversity emphasised.

REFERENCES

ANDERVONT, H. B., AND ED-   1942-43. *J. Nat. Cancer Inst.*, iii, 349.
   WARDS, J. E.
ANDERVONT, H. B., WHITE, J.,   1943-44. *Ibid.*, iv, 583.
   AND EDWARDS, J. E.
BECK, S., AND PEACOCK, P. R. .   1941. *Brit. Med. J.*, ii, 81.
DYER, H. M. . . . . .   1938. *J. Biol. Chem.*, cxxiv, 519.
EDWARDS, J. E., AND WHITE. J. .   1941-42. *J. Nat. Cancer Inst.*, ii, 157.
ELSON, L. A., AND HOCH-LIGETI,   1944. *Biochem. J*, xxxviii, Proc. Biochem.
   C.   Soc , p. x.
EMMART, E. W. . . . . .   1940-41. *J. Nat. Cancer Inst.*, i, 255.
GLYNN, L. E , HIMSWORTH, H. P.,   1945. *Brit. J. Exp. Path.*, xxvi, 326.
   AND NEUBERGER, A.
JACOBI, H. P., AND BAUMANN,   1942. *Cancer Res.*, ii, 175.
   C. A.
KENSLER, C. J., DEXTER, S. O ,   1942. *Ibid.*, ii, 1.
   AND RHOADS, C. P.

KENSLER, C. J., SUGIURA, K.,   1941.   *Science*, xciii, 308.
YOUNG, N. F., HALTER, C. R.,
AND RHOADS, C. P.

KENSLER, C. J., YOUNG, N. F.,   1942.   *J. Biol. Chem.*, cxliii, 465.
AND RHOADS, C. P.

KINOSITA, R.   . . . . . .   1936.   *Gann*, xxx, 423.

,, ...   1939-40. *Yale J. Biol. and Med.*, xii, 287.

KIRBY, A. H. M.   . . . .   1944.   *Nature*, cliv, 668.

,, ...   1945a. *Cancer Res.*, v, 673.

,, ...   1945b. *Ibid.*, v, 683.

LAW, L. W.   . . . . . .   1941.   *Ibid.*, i, 397.

MARUYA, H.   . . . . . .   1938.   *Trans. Jap. Path. Soc.*, xxviii, 541.

MICHAELIS, L., SCHUBERT, M. P.,   1939.   *J. Amer. Chem. Soc.*, lxi, 1981.
AND GRANICK, S.

MILLER, J. A., AND BAUMANN,   1945.   *Cancer Res.*, v, 227.
C. A.

MILLER, J. A., KLINE, B. E.,   1944.   *Ibid.*, iv, 756.
RUSCH, H. P., AND BAUMANN,
C. A.

MILLER, J. A., MILLER, E. C., AND   1945.   *Ibid.*, v, 162.
BAUMANN, C. A.

MILLER, J. A., MINER, D. L.,   1941.   *Ibid.*, i, 699.
RUSCH, H. P., AND BAUMANN,
C. A.

MIURA, K.   . . . . . .   1935.   *Bull. Assoc. franç. p. l'étude du cancer*, xxiv, 534.

OPIE, E. L. .   . . . . .   1944.   *J. Exp. Med.*, lxxx, 231.

ORR, J. W. .   . . . . .   1940.   This *Journal*, l, 393.

POTTER, V. R.   . . . . .   1942.   *Cancer Res.*, ii, 688.

SASAKI, T., AND YOSHIDA, T. .   1935.   *Arch. path. Anat.*, ccxcv, 175.

SMITH, M. I., LILLIE, R. D., AND   1943.   *U.S. Public Health Rep.*, lviii, 304.
STOHLMAN, E. F.

SUGIURA, K., HALTER, C. R.,   1945.   *Cancer Res.*, v, 235.
KENSLER, C. J., AND RHOADS,
C. P.

THOMSON, W.   . . . . . .   1936.   *J. Hyg.*, Camb., xxxvi, 24.

YOSHIDA, T.   . . . . . .   1932.   *Arch. path. Anat.*, cclxxxiii, 29.

,, ...   1934.   *Trans. Jap. Path. Soc.*, xxiv, 523.

Addendum. Since this paper was submitted for publication, it has been demonstrated by Kensler *et al.* (1946, 1947) that N:N-diethyl-4-aminoazobenzene (EAB) is de-ethylated by the liver of the rat when given orally in a rice-carrot diet. Thus absence of carcinogenic activity in the case of this azo-dye cannot be attributed to failure on the part of the liver to de-alkylate; de-alkylation *per se* would therefore seem not to be part of the carcinogenic process. Nevertheless it should be pointed out that EAB has only been tested in the rice-carrot diet in which AAB has always failed to induce tumours. It is possible that EAB would induce tumours of the liver in the rat if given orally in our diet R.D.1 or other specially favourable diet.

REFERENCES

KENSLER, C. J., MAGILL, J. W.,   1946.   *Arch. Biochem.*, xi, 376.
SUGIURA, K., AND RHOADS, C. P.

KENSLER, C. J., MAGILL, J. W.,   1947.   *Cancer Res.*, vii, 95.
AND SUGIURA, K.

THE CARCINOGENIC ACTION OF 2-ACETYL-AMINO-FLUORENE ON VARIOUS STRAINS OF MICE

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(PLATES VII AND VIII)

ARMSTRONG and Bonser (1944) described the effect of prolonged administration of 2-acetyl-amino-fluorene by stomach tube to CBA mice. The number of mice used was small, as the supply of the chemical was limited. Five epithelial tumours of the bladder—3 benign (2 of them with malignant areas) and 2 malignant—were ascribed to the treatment; hepatomata and a uterine sarcoma were regarded as possibly spontaneous. A comparison was made of the response of the mouse with that of the rat, as described by other authors.

It seemed important to extend this experiment by the use of a larger number of mice of different strains.

METHOD

In the present experiment the dose of the chemical was reduced to 0·2 c.c. of a 1·5 per cent. suspension in arachis oil given by stomach tube twice weekly, *i.e.* 6 mg. per mouse per week in place of 9 mg. in olive oil in the previous experiment. Treatment was continued until the animals seemed unlikely to survive longer, 77 weeks being the maximum period. As before, there was a considerable mortality from the shock of intubation in the early part of the experiment (50 mice, table I). The age at the commencement of treatment ranged from 9 to 16 weeks, 12 weeks being the age of choice.

EXPERIMENTAL RESULTS

Tumours occurred frequently in the bladder, liver and female breast, permitting a study of the influence of strain and sex upon their incidence. They occurred sporadically in the stomach, thyroid, renal pelvis and female generative tract, but were not observed in sites usual in the rat, such as the external auditory meatus, intestine, ureter, pancreas, brain or kidney.

Influence of strain

(a) *Bladder.* A graded series of changes was observed in the bladder epithelium, starting with mild patchy hyperplasia in the form of an increased number of layers of swollen cells and proceeding to infiltrating carcinoma without metastasis. The previous description (Armstrong and Bonser) requires little modification except in so far as more bladders at different stages were available for examination and therefore a greater variety of size and appearance of the tumours was seen. The largest malignant tumour measured 1·0 cm. in each diameter; it was a transitional-cell carcinoma with areas of keratinisation. The other carcinomas were of similar type. Mucus-secreting types were not seen. In one CBA female, treated for 62 weeks, abundant melanin pigment was found in patches in the cells of all the layers composing the hyperplastic epithelium (fig. 4).

TABLE I

Mice used and survival rate

Strain	No. of mice started			No. of mice dying between		Sex of survivors for 20 weeks and more	
	Male	Female	Total	20-49 weeks	50-77 weeks	Male	Female
CBA	12	13	25	1	17	9	9
IF	14	14	28	1	19	9	11
RIII	12	12	24	2	19	10	11
White label	22	23	45	9	15	12	12
Strong A	14	22	36	6	19	6	19

No strain was exempt from tumours of the bladder, but when the strains are arranged in descending order of susceptibility it is seen (table II) that there is a marked difference in incidence between the least and the most susceptible strains. In Strong A, only one of five papillomas showed early malignant change. This occurred in a female treated for 69 weeks. It should be noted (table I) that the number of males of this strain surviving 20 weeks of treatment was only 6. Since, when all the strains are considered together, the incidence of bladder tumours is higher in males than in females (table VI), it might have been higher in the Strong A strain had more males survived. By contrast, 12 of 14 CBA mice showed either malignant papillomatosis or infiltrating cancer.

(b) *Liver.* Changes of various kinds were seen. The most striking was marked swelling of the hepatic cell nuclei, with karyorrhexis; in some of the swollen nuclei one or more hyaline droplets were also seen. These changes were diffuse in some livers but patchy in others, when they were usually periportal in distribution. Collections of polymorphonuclear and mononuclear cells were sometimes seen in the portal tracts or were more diffusely distributed. Cirrhosis

TABLE II—*Bladder changes in mice of different strains surviving treatment for 20 weeks*

Strain	No of mice examined			Bladder normal		Hyperplasia		Papillomatosis					
	Total	Male	Female	Male	Female	Male	Female	Benign		Malignant		Total male and female (per cent)	Percentage of total papillomas which were malignant
								Male	Female	Male	Female		
CBA	18	9	9	0	1	2	1	0	2	7	5	78	86
IF	20	9	11	0	2	0	4	3	1	6	4	70	71
RIII	21	10	11	0	5	2	4	3	2	5	0	48	50
White label . .	23	12	11	0	4	7	4	2	0	3	0	22	60
Strong A . .	23	6	17	1	10	3	4	2	2	0	1	22	20

The χ^2 test shows that the possibility that the differences in incidence of papillomatosis in the various strains could have occurred by chance is <1 in 100.

TABLE III—*Liver changes in mice of different strains surviving treatment for 20 weeks or more*

Strain	No of mice examined			Swelling of hepatic nuclei	Collections of mononuclears and polymorphs	Bile ducts		Nodular hyperplasia of liver	Hepatoms				
	Total	Male	Female			Dilatation	Cholangioma		Benign		Malignant		Both types and sexes (per cent)
									Male	Female	Male	Female	
CBA . . .	15	7	8	0	3	1	0	0	3	5	3	0	73
IF . . .	18	6	12	11	0	5	1	0	0	5	3	1	44
RIII . . .	19	8	11	13	2	0	0	2	0	3	0	0	16
White label .	21	10	11	6	1	0	0	0	3	0	0	0	10
Strong A .	22	6	16	21	11	7	1	2	0	1	0	0	5

The χ^2 test shows that the possibility that the differences in incidence of hepatomas in the various strains could have occurred by chance is <1 in 100.

was not seen. There was mild dilatation of the small bile ducts in some livers; occasionally this was associated with proliferation, forming small insignificant cholangiomas. In a few livers there was multiple nodular hyperplasia. Hepatomas were also seen, conforming to the descriptions of other writers. A few had become malignant but no metastases were found. In the hepatic cells adjoining some of the hepatomas, was a golden-brown pigment. This gave a partial Prussian blue reaction but had not the characteristics of " ceroid ".

There was a strain difference in the incidence of these various changes (table III). Hepatoma was most common in the CBA strain, fairly common in IF and least common in Strong A. The strains could be arranged in the same descending order of frequency of incidence for both hepatoma and bladder papillomatosis (table II). Swelling of the hepatic nuclei was most marked in the Strong A strain and was completely absent in CBA. The significance of these differences is obscure. Spontaneous hepatomas occur not infrequently in the CBA strain (Strong and Smith, 1936 ; Gorer, 1940). In Leeds, 2 of 13 breeding mice (15 per cent.) dying between 52 and 85 weeks, i.e. within the experimental period, bore spontaneous hepatomas, in contrast with 8 of 24 mice (33 per cent.) dying between 85 and 117 weeks. As 73 per cent. of treated mice bore hepatomas (table III), most of which were larger than those arising spontaneously, there is little doubt that 2-acetyl-amino-fluorene is to be regarded as carcinogenic to the liver in this strain. No exact note has been made of the occurrence of spontaneous hepatomas in the other strains, but they must be very rare.

(c) *Breast.* Of the five strains tested, the females of three (RIII, Strong A and White label) are liable to develop spontaneous mammary cancer in descending order of frequency. Litter-mate controls of the experimental animals were not kept but observation of the strains in recent years has yielded the following incidence in mice living for 6 months or more :—RIII 71 per cent., Strong A 30 per cent., White label 3 per cent. (table IV). The latter figure is unduly low, as the average age at death of these mice was 10 months and tumours do not usually arise before 12 months or more. Table IV shows the incidence of breast cancer in females surviving treatment for 20 weeks or more. It is to be noted that in both the strains normally exempt, induced breast tumours occurred, the incidence being significantly higher in the IF than the CBA strain. Orr (1943) found that mice of both these strains developed breast cancer when methylcholanthrene was the carcinogen. The incidence in each of the three strains in which breast cancer occurs spontaneously was increased by the treatment in varying degree when compared with the incidence in the strains as a whole. As litter-mate controls were not available, no exact comparison can be made. Microscopically, the induced tumours did not differ from spontaneous tumours, except that half of those

in IF mice showed squamous metaplasia and keratinisation, a rather high proportion.

TABLE IV

Incidence of mammary cancer in females

Strain	Spontaneous mammary cancer in breeding females surviving for 26 weeks or more				Mammary cancer in female mice treated for 20 weeks or more			
	No of mice examined	No of cancers	Percentage incidence of cancer	Average age in weeks	No of mice examined	No of cancers	Percentage incidence of cancer	Average age in weeks
CBA	50	0	0	...	9	1	11	74
IF *	50	0	0	...	11	8	73	57
RIII	62	44	71	52	11	10	91	61
White label	31	1	3	60	12	7	58	48
Strong A	63	19	30	48	18	7	39	62

The χ^2 test shows that the possibility that the difference in incidence of breast cancer in the IF and CBA strains could have occurred by chance is 1 in 100.

* A female mouse of this strain given to Dr Orr was observed to have developed mammary cancer spontaneously at an age unknown (Orr, 1946)

(d) *Other organs.* Tumours in organs other than the bladder, liver and breast (table V) were so infrequent that a comparison of their incidence in the various strains could not be made. The four pedunculated squamous papillomas of the forestomach, occurring in

TABLE V

Incidence of changes in organs other than bladder, liver and breast

Site	Type of lesion	Strain	Sex	Weeks of treatment
Forestomach	Squamous papilloma	RIII	M	77
	,, ,,	CBA	F	74
	,, ,,	Strong A	F	69
	,, ,,	,, ,,	F	65
	Downgrowth of squamous epithelium at junction with glandular stomach (fig. 1)	White label	M	65
Thyroid	Adenocarcinoma with invasion of blood vessels (fig 5)	RIII	F	69
	Adenocarcinoma	Strong A	F	69
	Colloid goitre	CBA	F	70
Renal pelvis	Transitional cell carcinoma	White label	M	65
Cervix uteri	Squamous carcinoma	,, ,,	F	59
Vaginal outlet	,, ,,	RIII	F	69
Perineum	,, ,,	White label	F	66

mice of three strains and both sexes after prolonged treatment, were of simple keratinising type (figs. 2 and 3) and showed no tendency to malignant change. The stomachs of a great many aged normal mice of all the strains have been examined *post mortem* and no similar

tumours seen.   One White label male, treated for 65 weeks, showed a peculiar downgrowth of the squamous epithelium at its junction with the glandular stomach (fig. 1).   This was regarded as due to squamous metaplasia of the gastric glands at the junction.   The two adenocarcinomas of the thyroid occurred in a single lobe in non-goitrous glands in female mice treated for a long period of time. They were regarded as malignant and the presence of tumour tissue within blood vessels in one mouse confirmed this view (fig. 5).   One transitional-cell carcinoma of the renal pelvis was found in a male mouse and three tumours of the genital tract in female mice (table V). The squamous papilloma of the perineum had been present for many weeks before death and had grown slightly but was benign.

Influence of sex

(a) *Bladder*. From table VI it is seen that when all the strains are considered together the incidence of papillomatosis is significantly higher in males than in females.   The numbers are too small to permit consideration of each strain separately.

(b) *Liver*. There is no significant difference in the incidence of hepatomas in males and females (table VI).

TABLE VI

Sex differences of bladder and liver changes (all strains)

		Females		Males	
		No. of mice affected	Per cent.	No. of mice affected	Per cent.
Bladder	Normal, or hyperplasia of epithelium	42	67	15	32
	Papillomatosis	21	33	32	68
Liver .	No hepatoma	44	76	26	70
	Hepatoma	14	24	11	30

The χ^2 test shows that the possibility that the difference in incidence of bladder tumours between the sexes could have occurred by chance is <1 in 100, that of hepatomas is $1:4$.

The numbers of tumours in other organs are too small for estimation of any sex difference.

DISCUSSION

The results of the present experiment serve to confirm the view that 2-acetyl-amino-fluorene has a wider range of carcinogenic activity than any chemical thus far investigated and that the factors of species, strain and sex are of great importance in determining the site of origin of the induced tumours (table VII).   No satisfactory explanation of

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Fig 1 —White label male, treated for 65 weeks
Below, the squamous epithelium of the fore
stomach, above, the glandular epithelium, at
the junction, numerous epithelial pearls, which
appear to be gastric glands undergoing squamous
metaplasia ×80

Fig 2 —Strong A female, treated for 65
weeks Pedunculated papilloma of fore
stomach arising close to junction with
glandular portion ×4

Fig 3 —Low power view of papilloma seen in fig 2, showing the drawing up of the
muscularis mucosæ into the core and the well differentiated type of keratinis
squamous epithelium ×40

TABLE VII—*Analysis of tumours induced by 2 acetyl amino fluorene by various authors*

Author	Mode of administration	Species	Strain	Sex	Bladder	Liver	Breast	External auditory meatus	Intestine	Thyroid	Fore stomach	Other organs
Wilson, De Eds and Cox (1941) Bielschowsky (1944a)	Feeding Painting*	Rat Rat	Slonaker all ino Wistar albino Male	Male and female Male	10/39	3/39 5/5	3/39	8/39 1/5	1/39 (colon)			One tumour each in muscle, ureter, renal pelvis pancreas and lung
Bielschowsky (1946)	Feeding Feeding com bined with allylthiourea	Rat Rat	Wistar albino	Male Female Male	1/1 (papilloma) 4/5 (carcinoma)	39/46 10/17 4/5 1/5	3/40 21/47	8/40 8/17 2/5 4/5	3/40† 2/47‡	5/5 (adenoma) 3/5 (a enoma) 3/5 (carcinoma)		Several adenomas of lung One tumour in uterus and skin One tumour in kidney
Armstrong and Bonser (1944)	Oily solution by stomach tube	Mouse	CBA	Male Female		3/4 2/0						Two tumours in uterus
Bielschowsky and Green (1945) Lopez (1945) Dunn and Kessel (1945 46)	Feeding Feeding Feeding	Fowl Rat Rat	Rhode Island Red Buffalo	Male Male		2/4 2/0	2/4	2/4				One carcinoma of kidney One glioma of cerebrum
Bielschowsky (1946)	Feeding	Rat	Piebald	Male Female		10/13 10/25	1/2	10/13 20/25	1 (small intestine, containing Paneth cells) 0/13 (small intestine) 14/25 (small intestine) 2/25 (caecum)			One carcinoma of lung Two carcinomas of lung and one of eyelid
Helman and Meisel (1940)	Oily solution by syringe and curved needle into pharynx Ditto with add ition of aromatic aminoacids	Rat Rat	Wistar Wistar	Male Female Male Female		1/35 § 	2/35 §					Two adenocarcinomas and 3 adenomas of submaxillary gland, 2 parathyroid adenomas, one sarcoma of neck and one thyroid adenoma No tumours in 24 animals
Armstrong and Bonser (present communication)	Oily solution by stomach tube	Mouse	CBA IF RIII White label Strong A	Male Female Male Female Male Female Male Female	7/9 7/9 9/9 5/11 2/11 5/12 2/0 3/17	6/7 5/8 2/6 0/12 3/11 2/10 1/10	1/0 8/11 10/11‖ 7/12‖ 7/18‖			1/8 (carcinoma) 1/17 (carcinoma)	1/9 1/10 2/19	One squamous carcinoma at vaginal orifice One carcinoma of renal pelvis and cervix uteri, one papilloma of perineum

* A 4 per cent solution of 2 amino fluorene in acetone applied three weekly
† Site not stated except that one was in cæcum
‡ Site not stated
§ Sex of animals bearing tumours not stated
‖ These are mice liable to develop spontaneous mammary cancer

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these differences is yet available. It had been thought that, as the mouse strains could be arranged in the same descending order of frequency with regard to both bladder and liver tumours (tables II and III), the same hypothetical metabolite of 2-acetyl-amino-fluorene might be acting as the carcinogen as a result of splitting in the liver and excretion in the urine. But when the results of Wilson *et al.* and of Bielschowsky are considered, it is seen that the former authors obtained frequent bladder tumours and rare liver tumours in their strain of rat, whereas the latter author obtained frequent liver tumours and no bladder tumours in his two strains of rat. It is notable that, as Wilson *et al.* found, the tumours of the urinary tract are confined to the bladder epithelium except in the case of one mouse, in which a carcinoma of the renal pelvis was present. The significantly higher incidence of bladder tumours in male mice is in keeping with what is known of the spontaneous disease in man.

In view of the fact that CBA is the strain of choice for the induction of bladder tumours by acetyl-amino-fluorene, an attempt is in progress to induce bladder tumours in this strain with β-naphthylamine, already known to be carcinogenic to the bladder of the dog. Although this experiment has been in progress for a period long enough to expect tumours to have occurred, provided the dosage has been adequate, the bladders have all been normal, though hepatomas have been of frequent occurrence (Armstrong, Bonser and Stickland, unpublished observation). This result is especially disappointing, as a small susceptible domestic animal is needed for the investigation of industrial bladder cancer. The place of 2-acetyl-amino-fluorene in relation to the experimental induction of bladder tumours has been discussed by Bonser (1947).

In the report of a previous experiment (Armstrong and Bonser, 1944) doubt was expressed as to whether the hepatomas occurring in CBA mice should be regarded as spontaneous or induced. From the incidence obtained in the present experiment and from the fact that similar tumours were found in other strains not liable to develop spontaneous hepatomas, they are now regarded as induced. There is a vast literature on the hepatomas induced by other chemical carcinogens, especially the azo- compounds (summarised by Kirby, 1945). These resemble in all respects the hepatomas described here.

Bielschowsky (1944*b*) induced cancers of the thyroid in female Wistar rats when he combined the carcinogen with goitre-inducing doses of allylthiourea. Two cancers of the thyroid have been obtained in female mice treated with the carcinogen only and one colloid goitre in a female of another strain. Apart from this one example, 2-acetyl-amino-fluorene is not goitre-inducing.

Keratinising squamous papillomas of the stomach as well as squamous cancers have been induced by feeding with other chemical carcinogens, notably 3 : 4-benzpyrene, methylcholanthrene and 1 : 2 : 5 : 6-dibenzanthracene (literature reviewed by Beck, 1946).
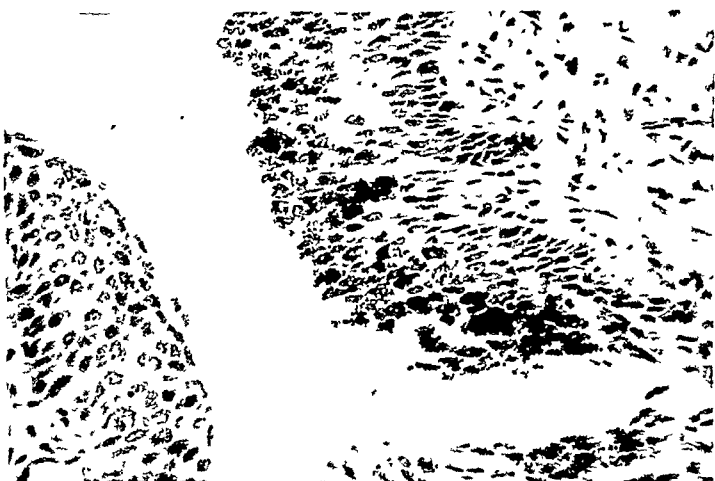
FIG 4—CBA female mouse, treated for 62 weeks Hyperplastic bladder epithelium containing granular masses of melanin pigment ×400
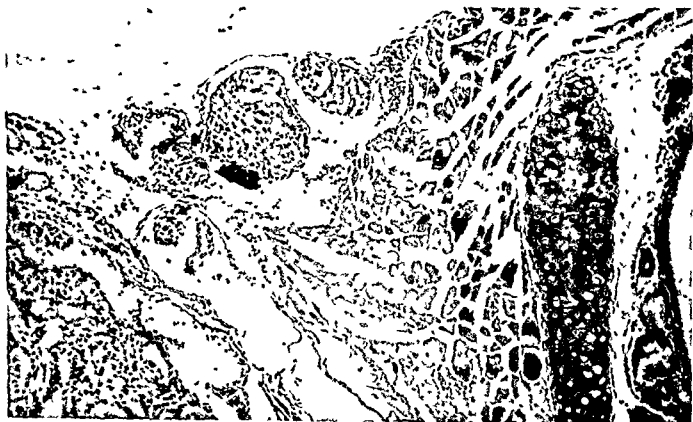
FIG 5—RIII female, treated for 69 weeks Adenocarcinoma of one lobe of thyroid gland, with invasion of blood vessels (upper margin to left of centre) ×120

The four papillomas described here remained benign during the period of the experiment.

SUMMARY

The results of administering 2-acetyl-amino-fluorene to mice of 5 strains by the gastric route are described. Tumours were observed in the bladder, liver, breast, forestomach, thyroid, renal pelvis and female generative tract. Marked strain differences with regard to bladder tumours, hepatomas and breast cancer were noted. A sex difference was observed in the incidence of bladder tumours but not of hepatomas.

A summary of the results obtained by other workers, who administered 2-acetyl-amino-fluorene to rats of various strains, and to fowls, is given in table VII.

REFERENCES

ARMSTRONG, E. C., AND BONSER, 1944. This *Journal*, lvi, 507.
G. M.
BECK, S. . . . . 1946. *Brit. J. Exp. Path.*, xxvii, 155.
BIELSCHOWSKY, F. . . . . . 1944a. *Ibid.*, xxv, 1.
    ,, ... 1944b. *Ibid.*, xxv, 90.
    ,, ... 1946. *Ibid.*, xxvii, 135.
BIELSCHOWSKY, F., AND GREEN, 1945. *Nature*, clvi, 780.
H. N.
BONSER, G. M. . . . . 1947. *Brit. Med. Bull.*, iv, 379.
DUNN, T. B., AND KESSEL, A. M. 1945-46. *J. Nat. Cancer Inst.*, vi, 113.
GORER, P. A. . . . . 1940. This *Journal*, l, 17.
HEIMAN, J., AND MEISEL, D. . . 1946. *Cancer Res.*, vi, 617.
KIRBY, A. H. M. . . . . . . 1945. *Ibid.*, v, 683.
LOPEZ, E. V. . . . . 1945. *Nature*, clvi, 296.
ORR, J. W. . . . . 1943. This *Journal*, lv, 483.
    ,, ... 1946. This *Journal*, lviii, 589.
STRONG, L. C., AND SMITH, G. M. 1936. *Amer. J. Cancer*, xxvii, 279.
WILSON, R. H., DE EDS, F., AND 1941. *Cancer Res.*, i, 595.
Cox, A. J., Jr.

Addendum. Since the present paper went to press, Dunning *et al.* have described the induction of neoplasms in 50 female rats of 5 strains by feeding with acetyl-amino-fluorene. Liver lesions, including cirrhosis and benign and malignant tumours, were frequent in all strains ; bladder and breast tumours were infrequent. The authors conclude that in spite of the small number of rats employed real constitutional strain differences in response to the chemical were manifested.

REFERENCE

DUNNING, W. F., CURTIS, M. R.,   1947. *Cancer Res.*, vii, 134.
AND MADSEN, M. E.

ATYPICAL AMYLOIDOSIS IN A CASE OF PLASMA-CELL MYELOMATOSIS

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(PLATES IX AND X)

THE term atypical amyloidosis was used by Lubarsch (1929) to connote instances of amyloidosis in unusual sites and occurring without obvious cause. In the cases which he described there was usually slight or no involvement of the organs usually affected with amyloid change, namely the liver, spleen, kidneys and suprarenals, while such organs as the skin, heart, lungs, serous membranes, and skeletal and intestinal muscles were particularly involved. The amyloid occurred either in the form of localised tumour-like masses or as a diffuse generalised infiltration of the affected tissues. It appeared that no sharp distinction could be made between the generalised and localised forms, and sometimes the most extraordinary combinations would be found.

At the same time Lubarsch attempted to classify the cases of atypical amyloidosis into three groups : (1) those involving mainly the skin and simulating scleroderma ; (2) those in which the dominant infiltration was in the muscles, simulating myatonia ; (3) those involving the tongue, simulating neoplasm.

Pick (1931) pointed out that there seemed to be a systematised type of atypical amyloidosis characterised by amyloid macroglossia combined with pseudo-myatonic stiffness of skeletal muscles and pseudo-sclerodermic hardening of involved areas of skin.

Since 1929 various authors have described cases belonging mostly to groups 1 and 3 above, in which a nodular atypical amyloidosis was usually manifested by macroglossia and in which no cause for the amyloidosis was determined (Paige, 1931 ; Perla and Gross, 1935 ; Robertson and Brunsting, 1936 ; Parkes Weber *et al.*, 1937 ; Barnard *et al.*, 1938). In such cases the liver and other favourite sites of the disease were only little or not at all affected. Staining reactions for amyloid in some of these instances have been pale or faint, especially metachromatic staining, and from such results there arose a suspicion that a compound closely allied to but not identical with amyloid may

have been present. The nodular character of the amyloidosis was likewise remarkable. The form in which the tongue was chiefly involved has been given the name of the Lubarsch-Pick syndrome because of the attention which these authors drew to the pathological and clinical aspects (Parkes Weber *et al.*).

It is noteworthy that a form of atypical amyloidosis may occur in association with multiple myeloma. In 1903 Parkes Weber reported on the association of amyloid deposits in the musculature of the tongue with diffuse plasma-cell myeloma of the bone marrow. His report was soon followed by that of Askanazy (1904), who described the local deposition of amyloid in the intestinal musculature in a case of plasma-cell myeloma. These were the first reported cases, in this country and on the Continent, of this remarkable association. Since then a small number of similar cases have been recorded, mainly in Germany and America : British references have been scant. Magnus-Levy (1931) stressed the relatively frequent association of these two rare conditions and, in summarising the literature on this subject, collected thirty-five cases of amyloidosis complicating multiple myeloma. In ten of these, localised masses of amyloid simulated tumours. It is surprising that in a review of 425 cases of multiple myeloma, Geschickter and Copeland (1928) do not mention the association with amyloidosis, though Snapper (1943) gives it brief mention.

One of the most striking features of amyloidosis occurring in association with multiple myeloma is its localisation. It may be generalised and found in almost every organ of the body, but more often it is localised in unusual sites such as the bone marrow, dura mater, abdominal wall, intestines, tongue and œsophagus, and in cartilage, connective tissue and muscles in and around the larger joints. In these atypical sites it is sometimes deposited in large tumour-like masses (Moschcowitz, 1936-37). This is in marked contrast with the usual distribution of amyloidosis when associated with chronic suppuration.

In myelomatosis, a localised massive deposit of amyloid within the muscular wall of the intestine has been found sufficient to cause intestinal obstruction (Bell, 1933). Robertson and Brunsting (1936) described the case of a man of 44 with amyloidosis of the tongue and amyloid nodules in the skin, œsophagus, stomach, small and large intestine and bladder. Mere traces were present in the kidneys, liver and spleen. Marrow softening in a vertebra with an increase in plasma cells, however, was the only bony lesion found. Rosenblum and Kirshbaum (1936) described a case in which a tumour-like deposit of amyloid was found in the sternum, and areas of myelomatous bone marrow were seen on microscopic examination of the ribs. Unfortunately there was no post-mortem, so that the extent of the amyloidosis and the question of whether the joints or skeletal muscles were affected could not be determined. Rosenheim and Wright (1933) reported a case with tumour-like amyloid deposits in the bone

marrow, liver and spleen consequent upon multiple myelomata. Fishberg (1939), also, mentioned a similar case which he saw with Dr R. H. Jaffé.

Case report

Clinical history

The patient, a man aged 58, was admitted to the Sheffield Royal Hospital complaining of gradual loss of weight for about 18 months. He had sought no early medical advice as he had felt quite well. About 11 months before admission he complained of aching pains in the legs, but only when sitting or lying down. There soon followed similar pains in both arms, but here the pain was continuous and was not aggravated by movement. The pain spread downwards from the shoulders to the finger-tips and there was a sensation of "pins and needles" which could be relieved by analgesics. For about 8 months before admission he began to be breathless on exertion and this gradually increased in severity. He had been treated by his own doctor with liver and iron for anæmia. There was only slight improvement in health and gradual, increasingly severe loss of energy was his chief complaint. Appetite had been good until a few weeks before admission, but now food induced sickness and vomiting. Lately this symptom had been frequent and persistent. The vomited matter was sour-tasting, dark brown fluid. During the time that these symptoms were at their peak he noticed a painless swelling, which gradually increased in size, on the front of his chest.

He had frequently passed dark brown or black stools, and had suffered occasional attacks of diarrhœa. Micturition was normal. He was a moderate smoker and beer-drinker.

On examination he was an emaciated elderly-looking man with a moist skin and cachectic yellowish pallor of the face and conjunctivæ. His tongue was clean and moist, there were no enlarged veins in the neck and the trachea was central. There was no lymph-node enlargement. There was a solid L-shaped swelling firmly adherent to the upper third of the sternum, approximately 1½ inches long by 2½ inches broad.

The arm and leg muscles were wasted, but tone and muscle power, and superficial and deep reflexes were normal; there was no œdema. The pulse showed no abnormality of rate, rhythm, volume or tension. The blood pressure was 120/70 mm. Hg. There was no cardiac enlargement or mediastinal deviation. There was a blowing systolic murmur at the apex and many coarse rales and rhonchi were heard over both lung bases. There was slight splenomegaly but the liver and kidneys were not palpable. There was no ascites.

Vomiting became persistent and troublesome and there were several small hæmatemeses. Dyspnœa became severe and the patient's pallor and sweating increased, and despite repeated small transfusions of blood he died 9 days after admission.

A previous aspiration biopsy of the lump on the sternum had shown it to be a plasma-cell tumour (fig. 1).

A blood count on admission showed Hb. 48 per cent., R.B.C. 2·7 million, C.I. 0·9, W.B.C. 9000. A differential count showed promyelocytes 1, myelocytes 8, metamyelocytes 15, neutrophil polymorphs 37, lymphocytes 39 per cent. No plasma cells were seen. There were 2 normoblasts per 100 white blood cells.

Post mortem findings

The mouth was edentulous but otherwise normal. There was no thickening of the bones of the skull or jaw. There was slight thickening of the left shoulder joint, but none of any other joint.

The abdomen contained no free fluid. There was gross distension of the ascending part of the duodenum and whole of the jejunum. The dilatation then decreased fairly sharply although there was no evidence of torsion or constriction. The wall of the affected gut was thickened and felt like wet wash-leather. There were numerous irregular shallow ulcers in the mucosa, situated mainly transversely both along the crests of the villi and in the depressions between them. They had slightly undercut edges and showed no obvious predilection for the lymphoid follicles. The serosa was rather dull, opaque bluish grey and showed no visible tubercles. The lower ileum appeared normal apart from slight congestion of Peyer's patches. The colon was full of tarry blood but was otherwise normal. Throughout the body of the stomach were a few minute shallow acute ulcers. These were also present in the duodenum just distal to the pyloric sphincter.

There was no enlargement of the mesenteric, portal or para-aortic lymph nodes. The spleen was rather large and there was patchy " sugar-icing " of its capsule. The cut surface was an even pinkish grey, and there were numerous pin-point red puncta throughout the pulp ; the malpighian bodies were not visible. Both adrenals were bulky, with an abundant watery cortex and medulla ; cortical lipoid was absent. There was no thrombosis of the superior mesenteric artery or portal vein. The kidneys showed a pale yellow cortex. The liver was pale and the trabecular pattern was marked by pale bulging periportal trabeculæ and sunken pink intervening areas. The bladder and prostate showed no abnormality.

The heart showed slight left ventricular hypertrophy. There was faint nodular fibrous thickening of the angles of the mitral valve and a sessile vegetation 0·1 cm. in diameter was situated on the left border of the posterior cusp. The aorta and coronary arteries showed negligible atheroma. There was a scanty clear effusion in both pleural cavities. The lungs, which were bulky, showed advanced bronchopneumonic consolidation of the whole of the right lower lobe and patchy early bronchopneumonia of the left lower lobe. The upper lobes were œdematous. The thyroid was small and pale. There was no enlargement of the hilar and aortic lymph nodes.

The whole of the manubrium sterni was enclosed within a completely patternless fleshy tumour up to 1·5 cm. thick, which had invaded the periosteum but not apparently the cortex. The medulla contained creamy purple marrow and similar marrow was present in the vertebral bodies and upper third of the right femur. There were no localised thickenings along the ribs. There were small sub-periosteal nodules of pale growth in the pelvic rami and the acetabula were so soft that they could be cut with a knife. The trabecular bone was here replaced by rather gelatinous tissue and there was consequent curving of the lateral pelvic walls due to the upward pressure of the femora.

The swelling of the left shoulder was caused by large masses of ill-defined soft white tissue like fish flesh which was closely infiltrating the tendons, muscles and joint capsule of the shoulder. Similar material surrounded the tendon of the psoas magnus muscle on both
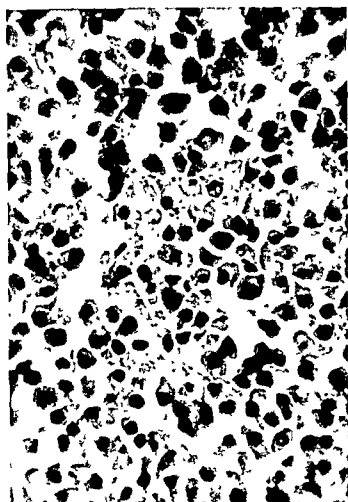
FIG. 1.—Tumour of sternum showing typical plasma cells. H. and E. ×600.

FIG. 2.—Amyloid nodules in the muscle and connective tissue around the shoulder joint. H. and E. ×85.

FIG. 3.—Amyloid infiltration and ischæmic necrosis of cardiac muscle. H. and E. ×85.

FIG. 4.—Amyloid infiltration of the wall of a coronary artery. H. and E. ×200.

ATYPICAL AMYLOIDOSIS

FIG. 5.—Early amyloid nodule in a tendon of the shoulder joint.   H. and E.   × 60.

FIG. 6.—Amyloid nodules in the mitral valve cusp.   Congo red.   × 60.

FIG. 7.—Amyloid infiltration of vessel walls in the subserosa of the jejunum.   H. and E. × 85.

FIG. 8.—Amyloid infiltration in the wall of a pulmonary arteriole.   H. and E.   × 85.

sides as it crossed the pelvis, and the lower part of this muscle was pale brown and œdematous.

The brain appeared normal apart from some adherent ante-mortem clot in the right transverse sinus.

Histological findings

Sections of nearly all the organs were stained with hæmatoxylin and eosin, hæmatoxylin and van Gieson, methyl violet and congo red. Amyloid, when present, was easily identifiable in H. and E.-stained sections, but excellent staining of this material was obtained with congo red (fig. 6), whether the amyloid was present as a diffuse infiltration of muscles and connective tissue or as discrete nodules. The metachromatic stain showed up the nodular amyloid deposits clearly but stained the more diffuse ones only faintly.

Jejunum and *ileum* showed gross amyloid change in the vessels (fig. 7) and in the submucosa and inner muscular layer, and there was amyloid infiltration with fibrosis of the outer muscular layer. The *large intestine* showed amyloid change in the vessels of the sub-serosa: there was fibrosis of the muscularis and homogeneous amyloid substance was present in the serosa. The *lungs* showed amyloid change in the pulmonary arteries (fig. 8) and inter-alveolar septa: there was a coexisting bronchopneumonia. The *heart muscle* showed two distinct features. There was amyloid infiltration of the media of the coronary vessels (fig. 4) causing obliteration of their lumen, which in turn had led to myocardial infarction. The more striking change was complete replacement of cardiac muscle bundles by discrete nodules of amyloid (fig. 3), having apparently no direct contact with vessels. The *mitral valve cusps* showed many large discrete amyloid nodules (fig. 6).

The "fish flesh" appearance of the *voluntary muscle* was caused in part by amyloid replacement of bundles of muscle fibres, in part by infarction, as many of the vessels were obliterated by amyloid deposited within their media and intima. The *connective tissue* and *joint capsule* from the shoulder showed masses of amyloid (fig. 2) and a tendon which was examined histologically showed notable infiltration (fig. 5). The *sternal tumour* was composed of polygonal plasma cells within a loose vascular net (fig. 1). The femoral marrow showed masses of plasma cells.

Liver, spleen and *kidneys* showed no amyloid apart from slight and rare involvement of the portal vessels. *Bladder* and *prostate* showed no amyloid, and it was detected in only very small amount in the suprarenal vessels. The *pancreatic, hilar* and *inguinal lymph nodes* were unaffected. The *pituitary gland* and *cerebrum* were normal. There was no amyloid in the walls of the *stomach* or *duodenum*, though the latter showed patchy interstitial fibrosis of the muscular coat.

Discussion

There are many more cases of atypical amyloidosis with no discoverable cause than cases showing an associated myelomatosis. Rosenblum and Kirshbaum, however, were of the opinion that a number of cases in which no cause for the amyloidosis can be found may show on closer inspection an underlying myelomatosis of the bone marrow. In their article on two cases of atypical amyloidosis Barnard *et al.* unfortunately furnish no report on an examination of the skeleton or on the state of the bone marrow, and in the light of previously published cases their statement that nothing is known as to any factors which may influence the development of atypical amyloidosis is not strictly correct. It is probable that some at least of the aches and pains around the joints and in the muscles in cases of myelomatosis may be attributable to amyloid infiltration of the joint capsules with involvement of joint spaces as described by Magnus-Levy. Yet these symptoms are often present, sometimes to a severe degree, without evidence of joint involvement (Geschickter and Copeland, 1931). Moschcowitz, however, discusses the not infrequently reported association of a chronic deforming arthritis with atypical amyloidosis.

Magnus-Levy maintains that the most obvious source for both amyloid and Bence-Jones protein is the bone marrow. This tissue is also responsible for the occasional occurrence of the high blood protein-euglobulin ratio found in myelomatosis. The assumption is that this protein may arise from destruction of the marrow by myeloma cells, with consequent release of much marrow protein. Bence-Jones proteinuria has, however, been absent in more than half the reported cases of associated atypical amyloidosis and myelomatosis. Experimentally the conversion of Bence-Jones protein into amyloid material by prolonged injection of this protein into the tissues of animals has not been possible. The detection of amyloid in tissues is almost entirely histochemical and depends upon the reaction of the substance to a metachromatic stain such as methyl violet or crystal violet, and to iodine and congo-red. Apparently amyloid is not always uniform in chemical composition, which may explain some of the bizarre staining reactions in atypical amyloidosis.

Summary

A new case of the rare systemic amyloidosis is described in which amyloid is deposited in a number of unusual sites. The organs chiefly affected were the heart, the smooth muscle of the small intestine, and the muscles, connective tissues and serous membranes in relation to large joints. Both nodular deposits and diffuse infiltration were present. In this as in some other reported cases, the atypical amyloidosis was associated with plasma-cell myeloma, in this instance

in the sternum and pelvis. Satisfactory histological staining of the amyloid was given with all the specific stains, though metachromatic staining showed best in the nodular lesions.

REFERENCES

ASKANAZY, M. . . . . 1904. *Verhandl. d. dtsch. path. Gesellsch.,* vii, 32.

BARNARD, W. G., SMITH, F. B. AND WOODHOUSE, J. L. 1938. This *Journal*, xlvii, 311.

BELL, E. T. . . . . 1933. *Amer. J. Path.,* ix, 393.

FISHBERG, A. M. . . . . . 1939. Hypertension and nephritis, 4th ed., *London*, p. 408.

GESCHICKTER, C. F., AND COPE-LAND, M. M. 1928. *Arch. Surg.,* xvi, 807.

" " " " 1931. Tumours of bone, *New York*, p. 430.

LUBARSCH, O. . . . . . . 1929. *Arch. path. Anat.,* cclxxi, 867.

MAGNUS-LEVY, A. . . . . . 1931. *Z. klin. Med.,* cxvi, 510.

MOSCHCOWITZ, E. . . . . . 1936-37. *Ann. Int. Med.,* x, 73.

PAIGE, B. H. . . . . . . 1931. *Amer. J. Path.,* vii, 691.

PERLA, D., AND GROSS, H. . . 1935. *Ibid.,* xi, 93.

PICK, L. . . . . 1931. *Klin. Wschr.,* x, 1515.

ROBERTSON, H. E., AND BRUNSTING, L. A. 1936. *Amer. J. Path.,* xii, 766.

ROSENBLUM, A. H., AND KIRSHBAUM, J. D. 1936. *J. Amer. Med. Assoc.,* cvi, 988

ROSENHEIM, M. L., AND WRIGHT, G. P. 1933. This *Journal*, xxxvii, 332.

SNAPPER, I. . . . . 1943. Medical clinics on bone diseases, *New York*, p. 181.

WEBER, F. PARKES . . . . 1903. *Med.-Chir. Trans.,* lxxxvi, 395.

WEBER, F. PARKES, CADE, S., STOTT, A. W., AND PULVERTAFT, R. J. V. 1937. *Quart. J. Med.,* xxx, 181.
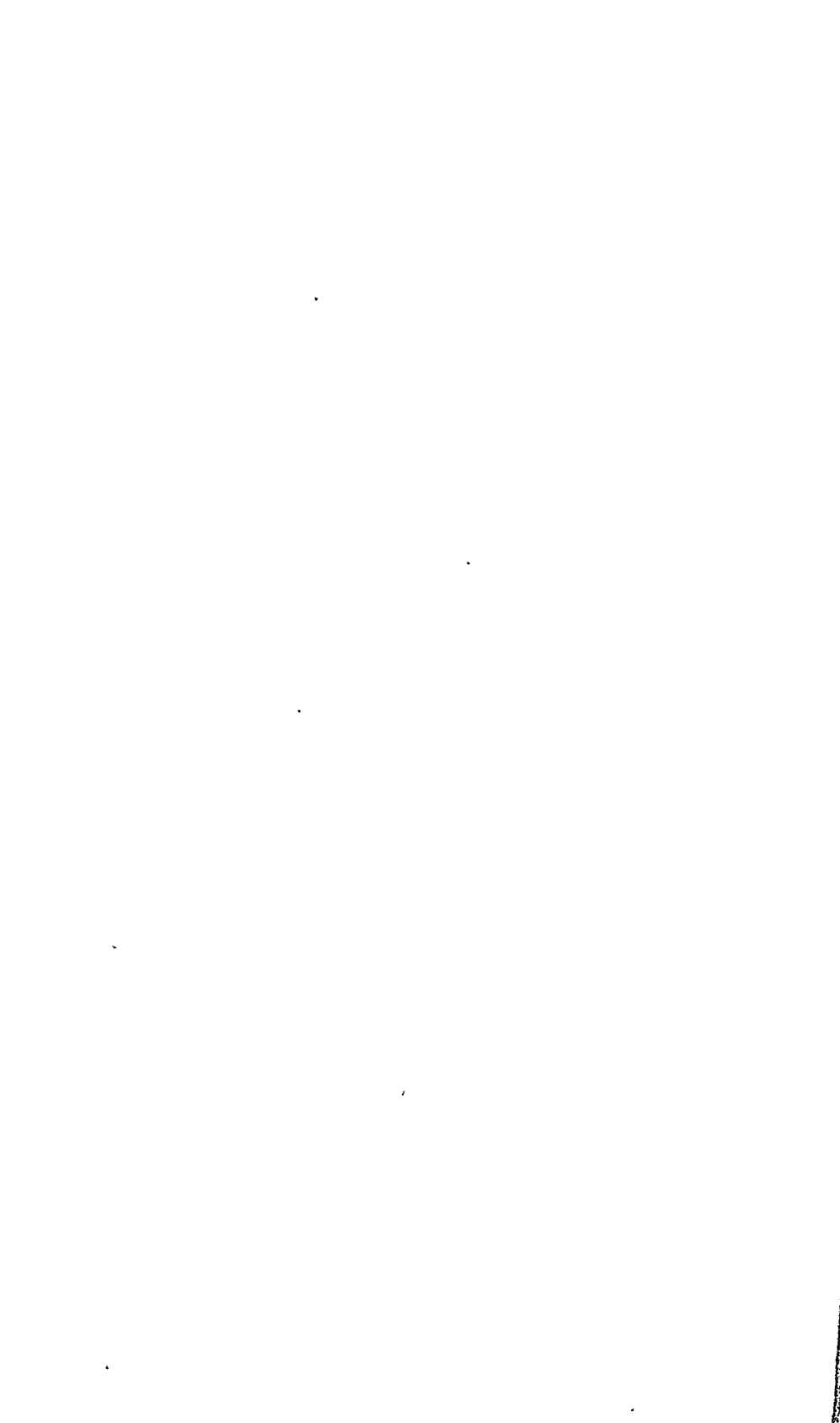
ON THE USE OF TISSUE-FREE MEDIA FOR THE PREPARATION OF BLACKQUARTER VACCINE : (I) CYSTEINE HYDROCHLORIDE BROTH : (II) ACID DIGEST OF LIVER AND MEAT

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IMMUNISATION against blackquarter with preparations of spore cultures in the form of dried and heated muscle pulp from an infected calf, and with natural and artificial aggressins, has given place in recent years to immunisation with formalin-treated cultures. This method, first introduced by Leclainche and Vallée (1925), and advocated by M'Ewen (1926), Karmann (1927) and others, has become the standard method of immunisation against the disease in many parts of the world. The immunity established is both antibacterial and antitoxic and is far superior to that established by means of natural or artificial aggressins.

To ensure massive growths for the purpose, use has been made of glucose broth containing pieces of autoclaved meat, pieces of sterile tissue or heart muscle of an inoculated guinea-pig (Cordier, 1926). These methods have the disadvantage that particles of the tissue used for creating anaerobiosis become mixed with the bacterial suspension. M'Ewen used diffusion shells containing minced liver in conical flasks of sucrose broth, and Sheather (1928) replaced the diffusion shells by porous porcelain cells. Among methods for preparing tissue-containing media, M'Ewen's procedure and Sheather's modification of it are the best for obtaining growth free from tissue particles. Among methods dispensing with the use of tissues, the catalytic device of McIntosh and Fildes (1916) is efficient with agar slants and plates and with small volumes of liquid media, but it is less useful for bulk preparation of vaccine. The central catalysing plant advocated by Boëz (1927) for growing anaerobes in a series of containers may be more useful for this purpose. Certain chemical substances have also been used to replace animal tissues in producing anaerobiosis. Reduced iron (Scott and Brandly, 1933), sodium hydrosulphite (Aristowsky and Minkewitsch, 1934-35) and levulinic acid all have reducing action. Certain substances containing a sulphydryl group, such as cysteine, glutathione and thioglycolic acid, are also powerful reducing agents. Ehrismann (1936) showed that ascorbic acid (vitamin C) can bring about growth of anaerobes.

But the literature dealing with chemical reducing agents in the growth of anaerobes is mainly academic. Except perhaps for sodium thioglycolate (Brewer, 1940) chemical reducing agents have not been used extensively for routine cultivation of anaerobes or bulk production of anaerobe vaccines.

PART I. CYSTEINE HYDROCHLORIDE BROTH

Hosoya (1925, quoted by Frei and Hall, 1931) demonstrated the use of cysteine for growth of anaerobes, and Frei and Riedmüller (1930-31) and Frei and Hall have grown anaerobes in solid and liquid media containing 0·3 per cent. cysteine hydrochloride. This medium is successful because the —SH group in cysteine has reducing properties capable of yielding a high reduction potential uninfluenced by the presence of its oxidised -S-S-form. The experiments described in this section concern the use of cysteine as reducing agent in media for routine production of blackquarter vaccine.

METHODS

Relatively high concentrations of cysteine hydrochloride render broth very acid and also turbid. A 12·5 per cent. solution of cysteine hydrochloride is neutralised by an equal volume of N.NaOH and the turbidity also disappears. For convenience, a solution of cysteine hydrochloride of known strength was sterilised by candle filtration and kept in a McIntosh and Fildes's jar under anaerobic conditions for addition to media as required. Such a solution retains its full reducing properties for a long period. Normal solutions of sodium hydroxide are usually sterile a few days after preparation, but such solutions were usually autoclaved and sealed with paraffined plugs. To prepare the medium, the cysteine solution and an equivalent quantity of NaOH were added to the broth. Sometimes a mixture of equivalent proportions of cysteine hydrochloride and NaOH solutions was added to media, the two solutions being autoclaved before or after mixing. Frequently, the required percentage of cysteine hydrochloride was added to broth, which was then adjusted to the required pH by addition of NaOH as usual and finally sterilised by heat.

EXPERIMENTAL RESULTS

Concentration of cysteine hydrochloride for growth of Cl. chauvoei

Expt. 1. Nine c.c. amounts of 1·0 per cent. glucose broth containing 0·05, 0·02, 0·01, 0·005, 0·002 and 0·001 per cent. respectively of cysteine hydrochloride were tubed. Duplicate tubes had a layer of paraffin. Two strains of *Cl. chauvoei* were used as seed, namely no. 14, an avirulent, and no. 26, a virulent strain, one drop of meat culture being seeded to each tube. Growth was read at 24 hours (table I).

Expt. 2. Cysteine hydrochloride was added to broth, with or without glucose, to final concentrations of 0·01, 0·025, 0·05 and 0·075 per cent. The pH was adjusted to 7·0. Ten-c.c. quantities of the media were sterilised at 120° C. for half-an-hour. When cooled

to room temperature, they were seeded with one drop of 18-hour meat culture. Three strains of *Cl. chauvoei* and one strain of *Cl. septicum* were used ; readings were made after 24 hours (table II).

TABLE I

Minimal effective concentration of cysteine hydrochloride in glucose broth for growth of Cl. chauvoei

| | Final concentration of cysteine hydrochloride (per cent) * | | | | | Cysteine neutralised | | | | |
| | before sterilisation | | | | | after sterilisation | | | | |
Strain	0 05	0·02	0 01	0·005	0·002	0·05	0 02	0 01	0 005	0 002
14 (P)	+	—	—	—	—	+	+	—	—	—
	+	—	—	—	—	+	+	—	—	—
26 (P)	+	+	+	+	—	+	+	+	+	—
	+	+	+	—	—	+	+	+	+	—

+ = growth ; — = no growth.
In series (P) the medium was covered by liquid paraffin.
* The sterilised cysteine was added to sterile medium in the final stage.

TABLE II

Minimal effective concentration of cysteine hydrochloride in broth and glucose broth

Strain			Final concentration of cysteine hydrochloride (per cent)			
			0 075	0 05	0 025	0 01
chauvoei 44 . . (G)			+	±	—	—
			+	+	—	—
,, 45 . . (G)			+	+	—	—
			+	+	—	—
,, 26 . . (G)			+	+	—	—
			+	+	±	—
septicum . . . (G)			+	+	±	—
			+	+	+	+

+ = profuse growth ; ± = moderate growth.
In series (G) the broth contained 1·0 per cent. glucose.

Expt. 3. To ascertain if growth was proportional to the amount of cysteine added, the growth was quantitatively estimated. Thirty-five-c.c. quantities of ordinary broth and of 0·5 per cent. glucose broth containing different concentrations of cysteine hydrochloride were made. The *p*H was adjusted (in most cases) to 6·8. Tubes were sterilised at 120° C. for half-an-hour and sown with 0·5 c.c. of meat culture of *Cl. chauvoei* 44 soon after cooling. The quantity of growth

after 48 hours was ascertained by weighing the centrifuged dried deposit (table III).

<div align="center">TABLE III</div>

Amount of growth in relation to concentration of cysteine hydrochloride

Medium	Concentration of cysteine hydrochloride (per cent.)				
	0·2	0·1	0·05	0·025	0·0125
Broth *p*H 6·8	7·5	4·3	3·9 (2·4, 2·2)	5·8	1·6
0·5 per cent. glucose broth *p*H 6·8	14·4	18·7	17·1 (14·3, 14·1)	16·7	15·5

Figures show dry weight of bacterial substance in mg.

Figures in brackets show dry weight of bacterial substance when the *p*H was 7·2 and 7·8 respectively.

With the inoculum used,' the least concentration of cysteine hydrochloride which could be relied upon to support growth of *Cl. chauvoei* under aerobic conditions was 0·05 per cent. With increasing concentrations of cysteine the resulting growth was greater, but with added glucose, within the ranges tried, the concentration of . cysteine hydrochloride made no difference to the quantity of the final growth.

<div align="center">*Optimum p*H *of cysteine hydrochloride broth*</div>

Broth containing cysteine hydrochloride at 0·05 and 0·075 per cent. was set at different levels of *p*H. The medium was autoclaved and used fresh. Media with a *p*H of over 7·2 usually showed a deposit of phosphates. If this happened, every lot of medium in that experiment

<div align="center">TABLE IV</div>

*Optimum p*H *for growth of* Cl. chauvoei *in* 0·075 *per cent.*
cysteine hydrochloride broth

Strain	*p*H 6·0	6·2	6·6	7·4	7·6	8·0
44	++	++	++	+	+	tr
45	++	++	+++	+	+	tr
26	++	++	++	++	+	tr
20	+	++	+	+	tr	tr
12	++	++	++	++	+	+

Growth estimated by visual examination after 8 hours at 37° C.; tr, etc. = degrees of growth.

was filtered and autoclaved a second time before being seeded from a 24-hour meat culture of one or more strains of *Cl. chauvoei*. The degree of growth was judged either by opacity after a short incubation or by weighing the dried centrifuged deposit, usually after a longer

incubation. Sample results in tables IV-VI show that the most favourable *p*H for *Cl. chauvoei* is about 6·8. However, it was found

TABLE V

Optimum pH of 0·05 per cent. cysteine hydrochloride broth autoclaved at 120° C. for half-an-hour

Initial *p*H of medium	.	.	.	6·2	6·6	7·2	7·4	7·7
Visual examination	.	.	.	+	+++	++	+	—
Nephelometer reading	.	.	.	5·36	15·0	3·0	1·5	1·0
Dry wt. (mg.) from 20 c.c. culture	.	1·5	4·0	0·9	0·4	0·3
Final *p*H of medium	.	.	.	6·1	6·5	7·0	7·4	7·7

Readings after 12 hours at 37° C.

— = no growth ; +, ++, +++ = increasing degrees of growth.

TABLE VI

Optimum pH for growth of Cl. chauvoei in 0·05 per cent. cysteine hydrochloride media

Broth	.	.	.	.	*p*H 6·3	6·6	6·8	7·1	7·5
					11·0	17·9	15·2	13·6	13·3
Glucose broth	.	.	.	*p*H 6·2	6·4	6·6	6·8	7·0
					45·2	45·5	42·0	60·5	65·8

Figures show dry weight in mg. of bacteria obtained after 48 hours at 37° C. from 120 c.c. of culture.

in earlier experiments that growth will take place even in extreme alkaline ranges in a sterile medium containing an excess of cysteine hydrochloride (0·3 per cent.) to which glucose is subsequently added.

Effect of different grades of heat on cysteine hydrochloride in broth. Frei and Hall stress the need to avoid changes in the composition of the cysteine through overheating. The effect of the following temperatures was tried : no heating, 100°, 105° and 120° C.

A 5 per cent. solution in water of cysteine hydrochloride at *p*H 6·8 was sterilised by filtration and 0·4 c.c. amounts were added to each of four 39·6 c.c. quantities of broth at *p*H 6·8 to yield a final concentration of 0·05 per cent. The first lot was unheated and the other three were heated at 100°, 105° and 120° C. for one hour, 30 minutes and 30 minutes respectively. When cool, each lot was seeded with 0·25 c.c. of supernatant of a meat culture of *Cl. chauvoei*. After 48 hours' incubation growth was ascertained as dry weight of bacterial substance (table VII).

TABLE VII

Amount of growth of Cl. chauvoei in relation to temperature of sterilisation.

Sterilisation by			
filtration	100° C. for one hour	105° C. for 30 mins.	120° C. for 30 mins.
5·9	4·0	5·8	4·7

Figures show weight in mg. of dry bacterial substance from 40 c.c. cysteine medium after 48 hours at 37° C.

Growth in the different lots was about the same and within the range of possible experimental error. Heating had no detrimental effects on cysteine under the conditions of the experiment.

Effect of heat on cysteine hydrochloride in broth at different H-ion concentrations. It was desired to ascertain if the selective pH range about 6·8 was an expression of optimal requirements or was due to loss of reducing property of cysteine hydrochloride when heated at other ranges of pH. Twenty-five-c.c. lots of 0·06 per cent. cysteine hydrochloride broth were adjusted to pH 3, 4, 5, 6, 7, 8 and 9 respectively. On heating at 118° C. for half-an-hour, media at pH 3, 4, 8 and 9 became turbid, those at 5, 6 and 7 remained clear. The pH of every lot was now adjusted to 7 by adding requisite quantities of HCl or NaOH. On neutralisation, media originally at pH 8 and 9 cleared; those at 3 and 4 still remained slightly opalescent. The media were again heated at 118° C. for half-an-hour and after cooling sown with 0·2 c.c. of an 18-hour meat culture of *Cl. chauvoei.* There was growth of the same degree in all the tubes 14 hours later. Thus under the experimental conditions cysteine hydrochloride did not lose its reducing power when heated at different pH levels.

Addition of glucose. Broth containing 0·05 per cent. cysteine hydrochloride was adjusted to pH 6·8 and glucose was added to 35-c.c. quantities to give concentrations of 0·0, 0·5, 1·0 and 2·0 per cent. respectively; the media were heated at 120° C. for half-an-hour and growth of *Cl. chauvoei* at 48 hours was estimated as dry weight of bacterial substance (table VIII). There was a threefold increase in

TABLE VIII

Addition of glucose to 0·05 per cent. cysteine hydrochloride broth, pH 6·8

Added glucose (per cent.)			
nil	0·5	1·0	2·0
6·5	18·7	18·5	19·0

Figures show weight in mg. of dry bacterial substance from 35 c.c. culture after 48 hours at 37° C.

growth when 0·5 per cent. glucose was added. Higher concentrations of glucose did not increase growth. The final pH in all lots containing glucose was 5·3, indicating that growth of *Cl. chauvoei* ceased at this pH without further utilisation of glucose. Fractional addition of alkali during incubation might of course promote continued growth.

Keeping quality of cysteine hydrochloride broth. Three 200-c.c. lots of broth were filled into conical flasks and heated at 120° C. for half-an-hour. The first lot contained 0·05 per cent. cysteine hydrochloride, the second 0·5 per cent. glucose, and the third both reagents in the concentrations stated; the pH was 6·8 and the flasks were left in the incubator until required. The longest time after its preparation that the medium will support growth of *Cl. chauvoei* was noted in four experiments.

Cysteine broth alone supported growth only if used fresh. With glucose added, it was useful for 24 and sometimes 48 hours. With added cysteine and glucose, the stored broth could be regenerated up to at least 5 days by heating it at 120° C. for half-an-hour immediately before use. Broth containing glucose alone never supported growth. It is interesting that glucose, which by itself could not support growth, could augment the growth value (tables III, VI and VIII) as well as the keeping quality of cysteine media.

Potency of anaculture and toxoid prepared by mixing cysteine hydrochloride cultures of Cl. chauvoei *and* Cl. septicum

As a medium for anaculture, 0·05 per cent. cysteine hydrochloride in 0·5 per cent. glucose broth was adjusted to *p*H 6·8 and heated at 120° C. for half-an-hour. In preparing anaculture at this Institute it has been the practice to mix 2-day and 14-day Noguchi cultures of *Cl. chauvoei* (two strains) and *Cl. septicum* (two strains) with the intention that the product should be polyvalent, antibacterial and antitoxic.

Proceeding in the same way with the experimental glucose-cysteine medium, it was desired to ascertain : (*a*) if there was any difference in the potency of the product from 2- and 14-day growths, and (*b*) if a layer of paraffin would alter the potency of the products. In both cases, at the stated times, formalin was added to a concentration of 0·3 per cent. and the cultures were left at 37° C. for a further 24 hours. The same four strains were used so that the cysteine medium might be compared with the routine Noguchi medium. After incubation for the stated times, portions of the formolised product were filtered to constitute toxoids.

Two hill bulls and one guinea-pig were vaccinated with each product, the doses being 5·0 c.c. for bulls and 2·0 c.c. for guinea-pigs. After 14 days, the animals were tested with a mixture of the four strains, bulls being given 50 m.l.d. and guinea-pigs 300 m.l.d. (table IX).

TABLE IX

Protective value of Cl. chauvoei *and* Cl. septicum *anacultures and toxoids from cysteine hydrochloride glucose broth*

Vaccine			Days grown	Paraffin layer	Bulls (50 m l d)	Guinea-pigs	
						(300 m l d)*	(30 m l d)
Anaculture .	.	.	2	Yes	0/2	2/2	0/2
Toxoid	.	.	2	,,	1/2	2/2	0/2
Anaculture .	.	.	2	No	0/2	2/2	0/2
Toxoid	.	.	2	,,	0/2	2/2	0/2
Anaculture .	.	.	14	Yes	0/2	2/2	0/2
Toxoid	.	.	14	,,	0/2	2/2	0/2
Anaculture .	.	.	14	No	0/2	2/2	0/2
Toxoid	.	.	14	,,	0/2	2/2	0/2
Unvaccinated	.	.	...	...	2/2	2/2	2/2

Numerator = no. died.
Denominator = no. tested.

* All vaccinated guinea-pigs tested with 300 m l d survived for more than 28 hours, whereas both unvaccinated controls tested with the same dose died in less than 14 hours.

Both anacultures and toxoids were found to be safe for the experimental animals. Potency tests in bulls showed that the

anacultures and·toxoids were equally efficacious, except possibly the toxoid from the two-day culture under paraffin. Fifteen of the 16 vaccinated bulls survived, while two control bulls died. In guinea-pigs, the results with 300 m.l.d. were uniformly bad, probably because this dose of test culture was too large. But whereas the control guinea-pigs died within 14 hours, all the vaccinated lived beyond 28 hours, suggesting a certain degree of protection. The test in guinea-pigs was therefore repeated, each animal receiving 2·0 c.c. of the product, which was followed in 14 days by a test dose of about 30 m.l.d. The result (table IX, last column) supports the finding for bulls and shows that the anacultures and filtrates were equally efficacious in protecting guinea-pigs against infection with certainly lethal doses of test culture. In our experience, natural aggressins and toxoids of *Cl. chauvoei* alone are less useful.

Potency of Cl. chauvoei *anaculture and toxoid prepared in cysteine hydrochloride broth*

It was surmised that the immunising property of the toxoids in the last experiment was due to the *Cl. septicum* toxoid, because Mason (1936) has shown that this toxoid can protect sheep against lethal doses of *Cl. chauvoei*. It was considered advisable, therefore, to test whether in cysteine medium sown with *chauvoei* sufficient toxin would be formed to produce a toxoid protecting against *chauvoei*.

Cysteine hydrochloride glucose broth as used in the last experiment was put up in three conical flasks in 50-c.c. quantities. As soon as the medium had cooled to room temperature, each flask was sown with 1·0 c.c. of a 24-hour meat culture of a virulent strain of *Cl. chauvoei*. One flask was formolised (0·3 per cent.) after two days' incubation and another after 14 days. After addition of formalin, the flasks were left at 37° C. for a further 24 hours. A portion of the contents of each flask was then filtered through a Berkefeld-N candle. A third flask sown with a heavy inoculum was formolised after 12 hours' growth in order to ascertain if there was any virtue in using a young culture, as with certain other bacterial vaccines. Groups of 8-16 guinea-pigs were given 1·0 c.c. of anaculture or 3·0 c.c. of toxoid. The animals were tested for immunity after 18 days with what was ascertained to be a lethal dose of calcium chloride suspension of washed spores of *Cl. chauvoei*. There was no significant difference in the mortality rates between the control group and the groups previously treated with filtrates; but the difference between the control group and the groups treated with anaculture was highly significant (table X). Thus *chauvoei* filtrates had no detectable protective properties. On the other hand, anacultures whether from 12 hours', 2 days' or 14 days' growth were efficient immunising agents. This is in agreement with Henderson (1932), who showed that somatic antigens play the major part in *chauvoei* immunity. It is preferable,

however, to use a 48-hour rather than a 12-hour culture, since growth is much more abundant at 48 hours.

TABLE X

Protective value of Cl. chauvoei *anaculture and toxoid prepared from cysteine hydrochloride cultures incubated for varying times*

Vaccine				Incubation for	Guinea-pigs
Anaculture	.	.	.	12 hours	0/8
,,	.	.	.	2 days	0/16
,,	.	.	.	14 ,,	0/16
Toxoid	.	.	.	2 ,,	4/8
,,	.	.	.	14 ,,	4/8
Unvaccinated	.	.	.	...	4/8

Numerator = no. died.   Denominator = no. tested.

DISCUSSION

For manufacturing blackquarter vaccine, there are obvious advantages in the use of a cysteine medium : it is clear, it can be sterilised in the final stage and it involves no handling beyond that required for bottling.

It is advantageous to use a mixed *chauvoei* and *septicum* anaculture, because it confers greater immunity than anacultures of *Cl. chauvoei* alone. It is also an advantage that the same vaccine can be used for sheep, in which *Cl. septicum* plays a predominant role.

Cysteine hydrochloride is now sold in India at Rs. 150 per lb. At first sight it may appear that cysteine vaccine is more costly than that made from Noguchi medium, but on calculation and making all allowances this is found not to be the case.

SUMMARY OF PART I

(1) The lowest concentration of cysteine hydrochloride that could be relied upon for aerobic growth of *Cl. chauvoei* in broth in conical flasks was 0·05 per cent. Growth was improved by the addition of 0·5 per cent. glucose.

(2) The optimum pH was 6·8, though growth would also take place in a slightly alkaline medium. There was no growth in a medium more acid than pH 5·6.

(3) Growth was equally good whether the medium was sterilised by filtration or by heat at 100-120° C. The usefulness of the medium was not reduced by heat sterilisation within a pH range from 3 to 9, provided it was finally adjusted to about pH 6·8.

(4) Cysteine hydrochloride broth must be used fresh, but if glucose is also present the medium is suitable for growth within at least the first 24 hours after preparation. Also, cysteine broth containing glucose could be regenerated within at least five days of its preparation by heating for half-an-hour at 120° C. just before use.

(5) Anacultures of *Cl. chauvoei* prepared from cysteine hydrochloride glucose broth are equally potent whether collected from 12-hour, 2-day or 14-day cultures. It is preferable, however, to use cultures two days old. *Chauvoei* toxoid alone has no appreciable protective properties, but if mixed with *septicum* toxoid it confers a high degree of immunity.

(6) The following procedure for preparing blackquarter vaccine from cysteine hydrochloride broth is recommended. Broth containing 0·5 per cent. glucose and 0·05 per cent. cysteine hydrochloride is adjusted to *p*H 6·8, filled into conical flasks to near the neck, heated at 120° C. for half-an-hour and sown soon after cooling with a 1 per cent. inoculum of *Cl. chauvoei* from an 18-hour meat culture. After 48 hours' incubation, an anaculture is prepared by the addition of formalin to a concentration of 0·5 per cent. and leaving the culture a further 24 hours at 37° C. It is preferable to add an equal volume of *septicum* anaculture prepared in the same manner.

PART II. ACID DIGEST OF LIVER AND MEAT

Less fastidious anaerobes such as *Cl. septicum* and *Cl. tetani* have been grown in relatively simple media such as glucose broth under a paraffin layer. It is well known that *Cl. chauvoei* will not grow in any such media.

It has been a general experience that by substituting liver extract for ordinary broth or pieces of liver for minced heart in Robertson's meat medium a more profuse growth of anaerobes is usually obtained. It was thought that this was perhaps due to the higher liver content of glycogen and perhaps of sulphur-containing proteins which may yield active -SH groups. But since a simple liver extract did not support growth of *Cl. chauvoei*, experiments were undertaken with liver digests. It was anticipated that some of the amino-acids released from the proteins by hydrolysis would contain sulphydryl groups, which are known to be powerful reducing agents. Ox liver suspended in twice its weight of water was therefore subjected to digestion with a commercial pepsin at 56° C. for 5 hours at *p*H 2·0 and to tryptic digestion according to Hartley (1922). The media were finally set at *p*H 6·8, and it was found that *Cl. chauvoei* had grown in the peptic but not in the tryptic digest. At the same time, it was noticed that the liver particles in the peptic-digest flask were little disintegrated and it was then found that the pepsin in use had become entirely inert ; hence it was concluded that a simple acid digest of liver would be a good medium for growing *Cl. chauvoei*.

METHOD OF PREPARING THE ACID-DIGEST MEDIUM

One kg. of minced tissue, 2 litres of water and 80 c.c. of 10 *N*. hydrochloric acid were left to digest at the required temperature (56° or 100° C.) for the desired period. The vessel was then placed at room temperature overnight and next

morning adjusted to *p*H 6·0 by the gradual addition of 10 *N*. alkali. This usually required about the same volume of alkali as of acid used for digestion. The *p*H was carefully checked during the addition of the last fifth of the alkali so that the *p*H should not go beyond 6·0. The medium was then steamed for 10 minutes and filtered through paper. The clean filtrate was adjusted to *p*H 6·6-6·8, distributed into conical flasks or large test-tubes (22 mm. diameter) as desired and steam-sterilised for an hour. The supernatant of an overnight meat culture serially diluted in saline was used for seeding.

Sometimes digestion was carried out with concentrated HCl. The minced tissue and concentrated acid (10 *N*.) were then well mixed, the water being added at the end of the stipulated period of digestion. The alkali required for neutralisation could be introduced with this water.

Experience showed that if the medium was not to be ruined certain precautions were necessary. Neutralisation of the acid must not be done while the digest is hot and the alkali should be added gradually, with shaking to ensure even mixing, either after or along with the water. The *p*H should not be allowed to rise beyond 6·8.

Replacement of liver by muscle in the medium

As meat (muscle) may be more easily procured than liver, it was desired to ascertain how much of the liver could be replaced by meat without reducing the growth of *chauvoei*. A mince of liver and veal was put up in different proportions. Water and 10 *N*. HCl were added as usual and after the suspensions had been left at laboratory temperature overnight as a matter of convenience, they were digested in the steamer at 93° C. for 5 hours. Media were prepared as described above from these digests and 35 c.c. amounts were put into large test-tubes, steamed and seeded with graded inocula (table XI).

TABLE XI

Replacement of liver by meat (muscle) in acid-digest medium

Seed (c.c.)	All liver	Proportion of liver to meat			All meat
		3 : 1	2 : 2	1 : 3	
1·0	16 ++++	16 ++++	16 ++++	16 ++++	16 +
0·1	20 ++++	20 ++++	20 ++++	16 ++	—
0·01	36 +±	36 +±	24 +++	24 +	—
0·001	—	—	36 +±	24 ±	—

— = no growth; ±, +, ++ etc. = increasing amount of growth.
Figures represent the time in hours at which growth was first seen.

Growth started first in the all-meat lot seeded with the largest inoculum; on inspection at 16 hours it was already bubbling gas while the others showed only turbidity. But this rapid growth came to an abrupt end and the final yield was the poorest of all the corresponding lots. At the other end, the lag period was relatively longer in media made from higher proportions of liver; and with the smallest inoculum no growth took place in them. The best medium was the one made from equal parts of liver and muscle.

Influence of concentration of acid, temperature and duration of digestion on the richness of the resulting medium

Digestion of mixtures of equal parts of liver and muscle mince was carried out at 56° or 93° C. in the presence of diluted or concentrated HCl for periods of 5 hours or 5 days. The media were constituted on the following day or after standing at room temperature for 5 days. Apart from these variations, the routine method was followed. The media were put up in 35 c.c. quantities in large test-tubes. They were used soon after constitution or after an interval of 5 days; in the latter case the media were regenerated by steaming for half-an-hour just before use. Three serial tenfold dilutions of meat culture were used for seed. The cultures were inspected at convenient intervals to note when growth started. The final growth was estimated by the nephelometer (table XII).

The best medium was obtained by digestion of the tissues in the presence of undiluted HCl at 93° C. for 5 hours on a single day. It did not matter if the medium was constituted soon after digestion or after staying at room temperature for 5 days. This medium was capable of regeneration on the 5th day, but it could also be regenerated up to 15 days according to experiments not detailed here. In general, however, it is much better to use the medium as soon as it is constituted. Two other media also proved good: the 5-day concentrated acid digest at 56° C. and the 5-day diluted acid digest at 93° C.

Potency of vaccine made from liver-muscle acid digest

An anaculture was prepared from a two-day growth of *Cl. chauvoei* in acid-digest medium. The medium was prepared from equal parts of liver and meat digested in the presence of concentrated acid at 93° C. for 5 hours. Five bulls and five guinea-pigs were vaccinated, each with 5 c.c. and 1 c.c. respectively of the vaccine. Five weeks later they were tested, together with five controls of each species, with 50 lethal doses of calcium chloride suspension of *chauvoei* spores. All the vaccinated bulls and guinea-pigs survived, but of the controls four bulls and all five guinea-pigs died. The vaccine was potent.

DISCUSSION

It was thought that the value of the liver digest in supporting the growth of anaerobes possibly depended on the presence of reducing sugars or protein disintegration products containing active -SH groups. But the nitroprusside test failed to reveal the presence of active -SH groups and reducing sugars could not be demonstrated by the Benedict test. Ascorbic acid, which can induce anaerobiosis, was not detected by dichlorophenol-indophenol. But on addition of cysteine to the medium a substance capable of reducing dichlorophenol-indophenol was formed, cysteine alone being incapable of reducing the dye. This might suggest the formation of ascorbic acid from dehydroascorbic acid by cysteine. Whatever the reducing substance, it is inactivated by heat in the alkaline range, and ascorbic acid is also inactivated under the same conditions. Further work is necessary before a valid explanation can be advanced how acid digests of liver support growth of anaerobes.

TABLE XII—*Value of acid-digest medium in relation to concentration of acid, temperature and amount of digestion*

Acid	Temperature	Period (h=hours, d=days)	Constituted (days after digestion)	Used (days after constitution)	0.01 c.c.		0.001 c.c.		0.0001 c.c.	
					hrs	growth	hrs	growth	hrs	growth
Dilute	56° C.	5h	1	0	12	±	12	±	12	±±
				5	—	—	—	—	—	—
			5	0	12	+	12	+	18	++
				5	24	++	—	—	—	—
		5d*	1	0	12	++	18	++	24	+++
				5	24	+++	—	—	24	+++
	93° C.	5h	1	0	12	+	12	+	18	++
				5	—	—	—	—	—	—
			5	0	12	++	18	++	24	++
				5	24	+++	24	+++	42	+++
		5d*	1	0	12	+++	24	+++	42	+++
				5	24	+++	42	+++	42	+++
Concentrated	56° C.	5h	1	0	12	±	12	+	12	+
				5	—	—	—	—	—	—
			5	0	12	+	12	±	18	++
				5	—	—	—	—	—	—
		5d*	1	0	12	+++	18	+++	20	+++
				5	18	+++	24	+++	24	+++
	93° C.	5h	1	0	12	+++	24	+++	24	+++
				5	40	+++	70	+++	30	+++
			5	0	12	++	24	++	24	+++
				5	24	+++	42	+++	42	+++
		5d*	1	0	12	++	20	++	24	±±
				5	42	±	42	±	—	—

— = no growth; ±, +, ++ etc. = increasing degrees of final growth. The figures represent time (hrs.) at which growth was first seen.

* The mixture was at the temperature stated for 5 hours daily for 5 days.

SUMMARY OF PART II

Cl. chauvoei was grown under aerobic conditions in a medium prepared from hydrochloric acid digest of ox liver. Growth was improved and the lag period reduced when half the liver mince exposed to digestion was replaced by muscle tissue. Five hours' digestion at 93° C. in the presence of concentrated acid was satisfactory; several days' digestion was required at 93° C. in the presence of diluted acid, or at 56° C. in the presence of concentrated acid. After digestion, the pH on addition of sodium hydrate must not exceed 6·8.

The medium remained usable for about fifteen days provided it was regenerated by heat before use.

REFERENCES

ARISTOWSKY, W., AND MINKE-WITSCH, J, 1934-35. Abstr. in *Zbl. Bakt.*, Abt. I, Ref., cxvi, 398.

BOËZ, L. 1927. *J. Bact.*, xiii, 227.

BREWER, J. H. 1940. *Ibid.*, xxxix, 10.

CORDIER, G. 1926. *C.R. Soc. biol.*, Paris, xcv, 848.

EHRISMANN, O. 1936. *Z. Hyg.*, cxviii, 544.

FREI, W., AND HALL, G. N. 1931. *Vet. J.*, lxxxvii, 259.

FREI, W., AND RIEDMÜLLER, L. 1930-31. *Zbl. Bakt.*, Abt. I, Orig., cxix, 282.

HARTLEY, P. 1922. This *Journal*, xxv, 479.

HENDERSON, D. W. 1932. *Brit. J. Exp. Path.*, xiii, 421.

KARMANN, P. 1927. *Z. Infektionskr. Haustiere*, xxxi, 226.

LECLAINCHE, E., AND VALLÉE, H. 1925. *Rev. gén. Méd. vét.*, xxxiv, 293.

MASON, J. H. 1936. *Onderstepoort J.*, vii, 433.

M'EWEN, A. D. 1926. *J. Comp. Path. and Therap.*, xxxix, 253.

MCINTOSH, J., AND FILDES, P. 1916. *Lancet*, i, 768.

SCOTT, J. P., AND BRANDLY, C. A. 1933. *J. Bact.*, xxvi, 1.

SHEATHER, A. L. 1928. *J. Comp. Path. and Therap.*, xli, 259.

THE BEHAVIOUR OF *SALMONELLA TYPHI* IN THE AGGLUTINATION REACTION

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THE somatic agglutinability of *Salmonella typhi* has for many years presented problems of considerable interest and importance to the bacteriologist. This organism is pre-eminent among salmonella types as a human pathogen and most of the work undertaken to elucidate the reasons for its pathogenicity has been concerned with analysis of its somatic antigenic structure, usually in relation to loss variation. Thus the development of rough colonial forms was found to be associated with loss of the surface polysaccharide antigen and exposure of the underlying antigen R, the variation being correlated with loss of virulence (Topley and Wilson, 1946, pp. 277 and 278). Weil and Felix (1920, quoted by Felix and Pitt, 1934a) first described the striking differences in O agglutinability which exist between different strains of *Salm. typhi* and commented upon the "remarkable" sensitivity of strain 901. This variability was satisfactorily explained by the classical investigations of Felix and his co-workers (Felix and Pitt, 1934 *a* and *b*; Felix, Bhatnagar and Pitt, 1934), who showed that hypo-agglutinability and mouse virulence were correlated with the presence in the cell of a heat-labile antigen, Vi, which masked the O antigen and protected the cell against opsonisation. Destruction of Vi antigen by physical or chemical means or the prevention of its development in the living organism by culture on phenol-agar or at temperatures well below the growth optimum, resulted in the unmasking of the O antigen and the development of O agglutinability comparable to that shown by strain 901 and other hypersensitive strains. Strain 901 was recommended by Felix as test organism for the titration of O agglutinins in the so-called qualitative Widal reaction for the serological diagnosis of enteric fever (Felix, 1924), and its non-motile variant, strain 901/0 (Felix, 1930), is now used almost universally for this purpose.

The work to be described began as a series of observations during the routine preparation of standardised agglutinable suspensions and diagnostic antisera for the use of military laboratories in India and elsewhere, and it was some time before any clear-cut line of investigation suggested itself. These and subsequent observations are published now with the object of defining rather than solving what promises to be an interesting and complex problem.

METHODS AND TECHNIQUE

Agglutinable suspensions.—Colonies from which seedings were made were carefully selected on grounds of smoothness, standard agglutinability and stability in saline. Young broth cultures were seeded to nutrient agar (Lab-Lemco 0·5 per cent., peptone 1·0 per cent, NaCl 0·5 per cent. and agar 2·5 per cent.) either in Roux bottles or large enamelled developing trays, and harvested after overnight incubation, control plates being spread from the broth culture as a check on the stability of colonial smoothness. Suspensions were made in 1 : 2500 mercuric iodide in 0·45 per cent. saline solution, buffered at pH 7·4 and containing 0·05 per cent. formalin. More recently I have used 0·25 per cent. formalin in distilled water, buffered as before (Bridges, 1943-44). Suspensions were adjusted to an opacity equivalent to 6000 million *Bact. coli* per ml., serologically standardised against homologous suspension obtained from the Standards Laboratory, Oxford, and tested for stability in 0·85, 3·4 and 6·8 per cent. saline. Suspensions of *Salm. typhi* strain 901 (referred to in the text as T/901/0), the permanently non-motile *Salm. paratyphi* A (C.M.P.L. no. 113/44) and C (C.M.P.L. no. 618/43), *Salm. typhimurium* strain Glasgow (Schütze, 1930) and *Salm. typhi* strain Vi(1) (Bhatnagar *et al.*, 1938) were not alcoholised. Suspensions of all other types were prepared from motile strains and alcoholised after harvesting to destroy flagellar agglutinability.

Treatment of suspensions by heat. Much of the work to be described resulted from a chance observation that heated suspensions of T/901/0 behaved very differently from untreated suspensions. Unless otherwise stated, the designation " heated " after an organism means that the suspension had been placed in a boiling water-bath (96° C.) for 15 minutes. Any other physical or chemical treatment of suspensions is described in detail in the text.

Preparation of antisera. Antisera were obtained by immunisation of rabbits with suspensions prepared as above except that suspensions of motile strains were heated in a boiling water-bath for two hours before injection. Four injections of 250-, 500-, 1000- and 2000-million organisms were given intravenously at intervals of 3-5 days, the animal being bled 5 days after the last injection. All O antisera were tested for the absence of H agglutinin before use.

Agglutination technique. Serial dilutions of serum were made in 1 : 2500 mercuric iodide in 0·45 per cent. saline solution buffered at pH 7·4, 1·0 ml. volumes and wide-bore (2 × ½ in.) tubes being used. One drop of suspension was added to each tube. After shaking, the rack was incubated for two hours at 37° C. and allowed to stand on the bench overnight. The end-point was taken as the highest dilution of serum not showing a definite " spot " of unagglutinated bacteria at the bottom of the tube. Whenever the end-point was in doubt, either because of an ill-defined spot or some apparent dispersion of bacteria in addition to a spot, the tube was shaken and its magnified image examined in a concave microscope mirror, the titre being taken as the highest dilution showing definite particulation. The great majority of sera were tested as soon as possible after bleeding, without inactivation or the addition of preservative.

Absorption technique. The requisite volume of suspension was centrifuged, the supernatant fluid discarded and the deposited bacteria re-suspended in the serum. The absorbed serum was centrifuged free of bacteria after one hour at 37° C. This method allows fractional absorption or absorption with two or more suspensions without significant dilution of low-titre sera.

EXPERIMENTAL FINDINGS

Preliminary bleeding of rabbits before immunisation appeared to show that the natural antibody titre was almost invariably much higher against T/901/0 than against other salmonella types. The

sera of a considerable number of rabbits were therefore tested for natural O agglutinins against T/901/O suspensions, both untreated and heated, and against suspensions of other salmonella types. All the rabbits tested were healthy and, so far as was known, had never been involved in a salmonella or other epizootic; they were derived from several different strains reared in widely separated parts of India. Of a series of 142 rabbits tested, the sera in 81 per cent. showed titres of 1 : 40 or higher against T/901/O, being above 1 : 160 in 32 per cent. The sera in 6 per cent. of rabbits yielded titres of 1 : 640 or 1 : 1280. In 92 rabbits of the series, the sera were titrated against T/901/O (heated), in 55 against *Salm. enteritidis* O, in 57 against *Salm. paratyphi* AO, in 58 against *Salm. typhimurium* O (strain Glasgow) and in 67 against a Vi-R strain of *Salm. typhi*. No serum showed a titre above 1 : 40, and the titres of between 91 and 100 per cent. of sera fell below this figure.

Two facts are clear. The first is that T/901/O is agglutinated by a majority of normal rabbit sera to a markedly higher titre than other salmonella types, although these types contain among them all the somatic antigens hitherto described for this strain of *Salm. typhi* (Bornstein, 1943). Analysis of sera giving titres of less than 1 : 80 for T/901/O has shown that the titre is almost always significantly higher against this strain than against the other types examined. A few sera showing exceptionally high T/901/O titres were tested in a dilution of 1 : 12·5 for the presence of agglutinins against the flagellar antigens of *Salm. typhi* and *Salm. enteritidis* with negative results.

The second is that when a suspension of T/901/O is heated its agglutinability by normal rabbit serum is so diminished as to conform to that shown by other salmonellas. This reduction in the agglutinability of T/901/O (heated) is not due to a simple physical change in a fraction or fractions of the somatic complex, whose ability to react is thereby diminished, as is shown by comparing the titres of a number of normal rabbit sera against the same suspension of T/901/O, untreated and heated. The results of such an experiment are shown in table I.

This experiment was controlled by a *Salm. typhi* O and two *Salm. enteritidis* O-immune antisera; in each case the titre against heated suspension was between one-half and one-quarter of that against untreated suspension. Of nearly 100 normal rabbit sera tested, the titre against T/901/O (heated) has never been found lower than 1 : 10. In table I, therefore, it may be assumed that the agglutinability of heated suspension by normal serum does not vary by more than four times (*i.e.* from 1 : 10 to 1 : 40). On the other hand, that of untreated suspension varies by as much as 128 times (*i.e.* from 1 : 10 to 1 : 1280) against the same sera. If the difference in agglutinability between untreated and heated suspensions were due to a fall in agglutination sensitivity of the normal O-antigen complex—or part of it—as the result of heating, the same batch of suspension would be expected to

show a fairly uniform fall in titre with every serum—as happened with the immune antiserum controls. But there was no such uniform fall. Moreover the O complex of *Salm. typhi*, containing the factors IX, XII$_1$, XII$_2$, XII$_3$ (Kauffmann, 1941), is known to be heat-stable, as are all the

TABLE I

Agglutinin titres of a series of normal rabbit sera against the same suspension of T/901/0, untreated and heated

Rabbit no.	* Titre against T/901/0	
	Untreated	Heated
109	640	40±
110	20	20
111	20	10
112	160	20
113	640	20
114	40	<20
132	80	40
136	320	<20
137	1280	<20
155	10	10

* In this and subsequent tables, titres are reported as the reciprocal of the serum dilution concerned.

O antigens of the Salmonella group with the exception of a component of antigen V (Kauffmann, 1936, quoted by Bornstein, 1943, p. 444), and can withstand 100° C. for two hours or more without deterioration. Suspensions of *Salm. paratyphi* A and C, of *Salm. typhimurium* and of several strains of *Salm. enteritidis* have failed to show any loss of somatic agglutinability after heating.

Natural agglutinins in the sera of species other than rabbits

The sera of 12 goats, 11 guinea-pigs, 4 chickens, 2 sheep and 2 dogs were tested for natural agglutinins against T/901/0 and other salmonella types. Unfortunately, T/901/0 (heated) suspension was not included as antigen in these tests. A significantly higher titre was always found against T/901/0 than against the other types. The lowest titres were shown by guinea-pig sera, which agglutinated T/901/0 in dilutions of from 1 : 10 to 1 : 80, but did not react with any of the other types in dilutions of 1 : 5 or over. Figures relating to the natural agglutinin content of human sera are difficult to assess accurately in India since all troops have received T.A.B. vaccine at some time or another, and previous infection or exposure to infection cannot be excluded in the civil population. Whether or not the behaviour of normal human sera is similar to that of rabbit sera, the sera of immunised, non-infected persons show a wide divergence between the titres against T/901/0 on the one hand and T/901/0 (heated) on the other. The behaviour of human sera is dealt with in detail later.

The antigenicity of " Vi-R" strains of Salm. typhi in relation to the agglutinability of T/901/0

Bhatnagar *et al.* described a strain of *Salm. typhi*, known as Vi(1), which behaved agglutinogenically as a pure Vi variant, being agglutinated to an insignificant degree by high-titre O antiserum after destruction of Vi antigen and giving rise to only a low titre of O agglutinins (1 : 200) on injection into rabbits. We have used this

TABLE II

*Comparison of titres against T/901/0, untreated and heated, before * and after immunisation of rabbits with Vi-R and O strains of* Salm. typhi

| Immunising strain | Assumed antigenic structure | Serum no. | Titres against suspensions of | | Salm. typhi, Vi(1) |
| | | | T/901/0 | | |
			Untreated	Heated	
Vi(1)	Vi-R	1	(40) 10,240	...	(5) 2560
		2	(40) 1280	(10) 10	(10) 640
		3 †	(160) 1280	(20) 10	... 160
		4	(10) 160	(10) 20	(5) 5120
		5	(80) 80	(20) 20	(5) 2560
		6	(160) 80	(20) 20	... 640
Rawlings	Vi-R	7	(160) 20,480	... 20,480	... 640
T/901/0	Pure O	8	(20) 10,240	(10) 2560	(40) 80
		9	(40) 40,960	... 10,240	...

* Initial titres are included in brackets above the final titres.
† Suspension heated at 96° C. for ½ hour before injection.

strain for several years for the production of Vi antiserum and the preparation of agglutinable Vi suspension. It has shown considerable variation in its O antigenicity, which initially was high, stimulating titres of from 1 : 1000 to 1 : 10,000, thereafter falling until it possessed no ability to produce O agglutinin at all. Several sera were tested against untreated and heated suspensions of T/901/0 following immunisation of rabbits with this strain when its O antigenicity was

falling. Some representative results, compared with those following immunisation with Vi-R strain Rawlings, which is supposed to be antigenically similar to Vi(1), and with T/901/0 are given in table II.

It is clear that the antigenic composition of Vi(1) is very different from that of Vi-R strain Rawlings; for Vi(1) tends to produce antibodies only against untreated T/901/0, but Vi-R Rawlings stimulates agglutinins which react equally with heated and untreated suspension. Table II illustrates and our experience confirms that heated suspensions of T/901/0 are nearly always agglutinated to approximately one-quarter of the titre against untreated suspension by homologous antiserum, irrespective of its titre.

It is improbable that the increase in T/901/0 titre following immunisation with Vi(1) is due to random variation in the titre of natural agglutinin. The sera of a number of rabbits were titrated at weekly intervals over a period of a month and in two cases after an interval of six months. Except in one of the rabbits tested after a six-month interval, little alteration in titre was found, suggesting that there is not a wide and comparatively rapid variation as there is with α agglutinin (Stamp and Stone, 1943-44; Francis and Buckland, 1945).

As might be expected, suspensions of Vi(1), which stimulate antibody production against T/901/0, are themselves completely inagglutinable by *Salm. typhi* O antiserum and normal rabbit serum if they are heated to destroy Vi antigen. On the other hand, such suspensions become increasingly O agglutinable when Vi antigen has deteriorated through age, or incubation at 37° C. This agglutinability disappears on boiling the suspension for a few minutes.

Properties of the natural antibody

(1) *Type of agglutination.* With the majority of normal rabbit sera showing fairly high titres, agglutination of T/901/0 was coarsely granular and accompanied by complete clearing of the supernatant fluid. Partial or complete inhibition of agglutination was frequently observed up to 1 : 20 or 1 : 40 dilution of the serum. Agglutination of heated T/901/0 suspension was invariably in extremely fine particles, often visible only on magnification and accompanied by little or no clearing of the supernatant fluid. The same differences in degree and quality of agglutination were observed when Dreyer's instead of Felix's agglutination technique was used, except that the titre against untreated T/901/0 was usually a little lower.

(2) *Specificity and absorbability of the antibody.* Unsuccessful attempts were made to produce an antiserum which would agglutinate untreated but not heated T/901/0 suspension by absorbing normal rabbit serum with suspensions which had been boiled for periods up to 2½ hours or with suspensions of several strains of *Salm. enteritidis.*

Although boiled suspension was readily capable of removing all agglutinin from the serum, differential technique showed that absorption was rather less efficient in its later stages than with untreated suspension. Similarly, absorption with inagglutinable alkali-treated suspension (see below) removed all agglutinin against untreated suspension. Absorption of normal rabbit serum with suspensions of salmonellas other than *Salm. typhi* and *Salm. enteritidis* showed that, in the great majority of sera, the antibody possessed a high degree of specificity for *Salm. typhi*, its titre being reduced to only a slight extent or not at all by very dense suspensions of heterologous types. Yet although specificity is the rule it is not invariable, for the antibody titre of a few of the sera was considerably reduced by absorption with *Salm. paratyphi* A(I, II, XII_1, XII_3) and B(I, IV, XII_1, XII_2), *Salm. durazzo* (II, XII_1, XII_3) and *Salm. reading* (I, IV, V, XII_1, XII_2), but never by *Salm. paratyphi* C(VI, VII), *Salm. poona*, *Salm. gaminara* or any salmonella not sharing a somatic antigen with *Salm. typhi*. Whether this is due to a qualitative difference in the rabbit sera or in the absorbing suspension is not known.

It is a frequent finding that so-called " natural " antibodies require a much larger volume of absorbing suspension to exhaust them from a serum than do antibodies resulting from immunisation (Topley and Wilson, 1946, p. 1087). Absorption experiments were performed to determine if antibody against T/901/0, untreated and heated, could be removed from normal rabbit serum as easily as from immune serum of the same titre. Constant volumes of serum were absorbed with geometrically increasing numbers of T/901/0 and the titres of residual antibody estimated. The initial fall in titre of two normal rabbit sera was found to be more rapid than that of a *Salm. enteritidis* O antiserum similarly absorbed. But at a level below the titre of the unabsorbed serum against heated suspension the rate of fall was much slower. For example, with one serum 6000 million organisms sufficed to reduce the titre against untreated T/901/0 suspension to 1/32 of its original value—the end-point of agglutination against heated suspension—without significantly affecting the T/901/0 (heated) titre. Reabsorption with the same number of organisms was required to halve this titre, that for heated suspension now running parallel. Thus natural antibody against T/901/0 shows an unusual degree of absorbability up to the point where the titre corresponds to that against heated suspension, but thereafter absorption becomes increasingly difficult, the titres for untreated and heated suspensions coinciding.

(3) *Heat-stability of the antibody.* A number of normal rabbit sera were titrated, both before and after heating at 60° C. for 30 minutes, against T/901/0, untreated and heated, and several other salmonella O suspensions. Most of the sera after heating showed no fall in titre against untreated T/901/0, but titres dropped appreciably against T/901/0 (heated) and the other salmonella suspensions tested.

Some fall in titre against untreated T/901/0 was noted in only a few cases, and it was never greater than one tube.

(4) *Fractionation of normal sera.* A few sera were fractionated with ammonium sulphate. All the antibody against untreated T/901/0 suspension was present in the fraction precipitated by 50 per cent. $(NH_4)_2SO_4$ and half was present in the 35 per cent. precipitable fraction.

(5) *Relationship of the antibody to normal intestinal flora.* The fæces of four rabbits whose sera gave high titres against T/901/0 were examined for the presence of Salmonella organisms with negative results. The fæces of one such rabbit was plated on desoxycholate-citrate medium, MacConkey's agar and blood-agar. Ten different varieties of organism were isolated and a suspension prepared from each. One of these suspensions proved auto-agglutinable, but of the remainder only one was agglutinated to a titre of more than 1 : 20 by the rabbit's serum. The serum was then heavily absorbed with each of the ten suspensions without lowering at all its titre against T/901/0.

The effect of differential heating and of agencies other than heat on the agglutinability of T/901/0

T/901/0 suspension was divided into 5 portions each of which was heated at 50, 56, 60, 75 or 85° C. for one hour. Each suspension was then put up against a series of dilutions of normal rabbit serum (no. 113, table I) along with controls of untreated suspension and suspension boiled for 15 minutes. Suspensions heated at 50° C. gave the same result as the unheated control, being agglutinated cleanly by the serum to a titre of 1 : 640. Those heated at 75 and 85° C. gave results similar to boiled suspension, the serum titre being reduced to 1 : 20. Suspensions heated at 56 and 60° C. showed a marked deterioration in the quality of agglutination for serum dilutions of 1 : 40 to 1 : 640 inclusive. There was little clearing of the supernatant fluid, the agglutinated particles were very much finer than those in the untreated control and the aggregates at the foot of each tube gave the impression of a diffuse " spot ", thinning out at the periphery to a muddy particulation. Appearances, in fact, were intermediate between the clear-cut agglutination of the untreated control and the clean, compact " spots " of the heated control. Heating at temperatures of 56° C. and over, therefore, affects qualitatively the agglutinability by normal rabbit serum of T/901/0, until at 75° C. it is destroyed after one hour.

Treatment with 5 per cent. HCl, dilute alkali, alcohol in concentrations below absolute, saturated chloroform water or acetone destroys or greatly diminishes the agglutinability of Vi antigen without affecting that of the heat-stable O antigens. The effect of these reagents on the agglutinability of T/901/0 by normal rabbit serum was investigated. The suspension to be tested was divided into

a number of fractions, each of which was treated in one of the following ways :—

(1) Concentrated HCl was added to give a final concentration of 5 per cent. After 48 hours at room temperature (or at 4° C. during hot weather) the *p*H was adjusted to neutrality, the suspension washed three times and re-suspended in the original volume of saline (Felix and Pitt, 1936).

(2) One twenty-fifth the volume of *N*/1 NaOH was added. After four hours at room temperature the *p*H was adjusted to neutrality and the suspension washed and re-suspended in saline to the original opacity (Felix, 1945, personal communication).

(3) The suspension was centrifuged and the deposited bacteria re-suspended in saturated chloroform-saline. After one hour at 37° C. the suspension was washed and re-suspended in the original volume of saline.

(4) The suspension was centrifuged and the deposited bacteria re-suspended in pure acetone, in absolute alcohol, or in 75, 50 or 30 per cent. alcohol. After standing at room temperature for 48 hours the suspensions were washed and re-suspended in saline as before.

Suspensions so treated were tested against a normal rabbit serum and serum from a rabbit after immunisation with *Salm. typhi* strain Vi(1), giving titres of 160 and 10, and 1280 and 10 respectively against untreated and heated T/901/0. Similar results were obtained with both sera. Agglutinability was unaffected by treatment with 5 per cent. HCl, chloroform-saline, acetone and absolute and 30 per cent. alcohol. Treatment with 75 per cent. alcohol reduced agglutinability to one-quarter that of untreated suspension. Treatment with alkali was variable in effect, reducing the agglutinability of some suspensions to the same extent as heating, while that of other suspensions was unaffected. The effect of 50 per cent. alcohol was also variable and depended on the batch of T/901/0 suspension treated, agglutinability frequently being more markedly reduced than by 75 per cent. alcohol but occasionally remaining unaffected. In control experiments agglutinability by *Salm. enteritidis* O antiserum was never reduced to more than one-half by treatment.

The agglutinability of T/901/0 by human sera

Since T/901/0 is almost universally used as *Salm. typhi* O antigen in the Widal reaction, its behaviour in relation to human sera is of particular interest in view of the above findings. It has previously been pointed out that normal human sera cannot be obtained from Army personnel. Results obtained with 15 sera from healthy, T.A.B.-inoculated persons and from immunised persons suffering from enteric and other diseases are shown in table III. The titres of these sera against *Salm. paratyphi* AO and three strains of *Salm. enteritidis*

possessing different combinations of the components of antigen XII (Kauffmann) are included in the table.

It is clear not only that the divergence in titre against untreated and heated suspensions of T/901/0 and the lack of any proportional relationship between the two is at least as marked with human as with rabbit sera, but also that the titres against one of the two *Salm. enteritidis* strains 511/44 and 12316/JT, in which antigen XII$_2$ is absent or recessive, correspond roughly to those against T/901/0 (heated). It is hoped in a subsequent paper to deal more fully with the bearing of these findings on the evaluation of the Widal reaction

TABLE III

Agglutinin titres of human sera against various salmonella suspensions

Serum	Condition	Time since last T.A.B. inoculation (months)	T/901/0 IX, XII₁, XII₂, XII₃		A/113/0 I, II, XII₁, XII₂	Salmonella enteritidis O		
						511/44 IX, XII₁, XII₂	570/44 IX, XII₁, XII₂	12316/0 IX, XI (XII₁) XII₂
			Untreated	Heated				
W. H.	Healthy	24	1280	<10	<20	<20	<20	<20
M. B.		4	640	40	<20	40	<20	40
K. M.		14	20±	<10	<10	<10	<10	...
R. D. W.		14	320	<10	20±	10	10	<10
J. N.		14	320±	<10	80±	<10	<10	...
K. L.	Pleurisy	Not more than 18	160±	20±	10	...	...	...
384B	Endemic (murine) typhus	?	160	20	20 Tr.	<20	20 Tr.	...
385B		?	320	<20	<20	40	20	40
Proctor	Typhoid fever	?	1280	160	<20	80	<40	160
Kibble		6	640	160	10	160	40	160
Stock		8	1280±	20	20	40	20	80
Russell	*Salm. paratyphi* A infection	? 3	320	<20	20	<20	<20	...
Beamish		18	320	<20	160±	20	<20	...
Walton		3	640 Tr.	80	80	160	<40	...
Warne		2	320	<20	<40	<10	<10	...

in inoculated persons. In view of the similarity in behaviour of normal rabbit and human sera, some of the properties of the antibody against untreated T/901/0 in human serum W.H. were examined in more detail. As with the majority of fairly high-titre normal rabbit sera, untreated suspension was agglutinated in coarse granules with clearing of the supernatant fluid, and zoning was marked. Zoning was also observed with several other human sera. The antibody was readily absorbed by T/901/0, untreated and boiled for 2½ hours, and by suspensions of all three strains of *Salm. enteritidis* listed in table III. It was absorbed only slightly or not at all by *Salm. paratyphi* A, *Salm. durazzo*, *Salm. reading* and combinations of these types. Absorption with a " first generation " rough variant of *Salm. enteritidis*

strain 12316/JT reduced the titre of serum W.H. from 1:1280 to 1:160 but failed to remove any antibody from human serum M.B. This incongruity will be referred to later. Heating serum W.H. at 60° C. for 30 minutes did not appreciably reduce its T/901/0 titre. The serum was not fractionated. As with rabbit sera, the agglutinability of T/901/0 by serum W.H. was variably reduced to that of heated suspension by alkali treatment, and greatly diminished by contact with 50 per cent. and 75 per cent. alcohol. Human serum W.H., therefore, does not differ markedly from rabbit sera in the properties investigated.

The behaviour of freshly isolated strains of Salm. typhi

The agglutinability of ten freshly isolated strains of *Salm. typhi* received from other laboratories for routine confirmation of bacteriological diagnosis was compared with that of T/901/0. Such strains are complicated by the presence of Vi antigen, which masks somatic agglutinability to a variable degree. Suspensions were therefore tested untreated, after simple heating, after treatment either with 5 per cent. HCl or saturated chloroform-saline to destroy Vi agglutinability, and after chloroform- or HCl-treatment followed by heating. Chloroform treatment proved superior to HCl treatment, which showed a tendency to produce saline instability in some strains, but the results of both methods were substantially the same. These are shown for 5 strains, compared with those given by T/901/0, in table IV.

With the untreated suspensions there is a fairly close direct relationship between the titres to which they are agglutinated by human serum W.H. and *Salm. enteritidis* strain 511/44 antiserum and, as might be expected, these titres are about inversely proportional to the amount of Vi antigen present as judged by titre and quality of agglutination by pure Vi antiserum. Reference to the figures for untreated T/7/46, T/2/46, T/8/46 and T/16/46 suspensions, in that order, will make this clear. As with T/901/0, simple heating of these suspensions destroyed their agglutinability by human serum in every case. It has frequently been stated, and is generally assumed, that boiling not only destroys Vi agglutinability but also unmasks O antigen so that the organism becomes readily agglutinable by O antiserum. This has not been my experience. Most strains tested have shown a marked fall in sensitivity to O antiserum after heating (e.g. T/2/46 and T/16/46). A marked rise in titre was observed with only one strain (T/7/46).

Treatment with chloroform-saline raised the agglutinability of all the strains by both serum W.H. and *Salm. enteritidis* O antiserum to approximately that of T/901/0. Boiling the chloroform-treated suspensions did not impair agglutination by *Salm. enteritidis* O antiserum against strain 570/44, and sensitivity to strain 511/44 antiserum was reduced by not more than four times, often only slightly or not at all. These findings are in keeping with those observed for T/901/0

(heated) suspensions.   Results with human serum W.H. were unexpected, however, for the difference in sensitivity between untreated and heated chloroform-treated suspensions, instead of being uniform, varied from 4 to 64 times with different strains.   This variability is not correlated with either the quantity or quality (phage type) of Vi antigen in the strain.

TABLE IV

Agglutinability of freshly isolated strains of Salm. typhi *by human and immune rabbit serum*

Strain	* Suspension treated with	Human serum W.H.	Immune rabbit serum *versus*		Salmonella typhi Vi (pure)
			Salmonella enteritidis strain		
			570/44 (IX, XII₁, XII₂)	511/44 (IX, XII₁, XII₂)	
T/901/0	...	1280	51,200	12,800	<10
	Heat	<10	51,200	6400±	...
T/2/46	...	40	...	800	160+++
	Heat	<20	...	200 Tr.	<10
	CHCl₃	1280	51,200	12,800	<10
	CHCl₃+heat	20	51,200	12,800 Tr.	...
T/7/46	...	10	...	100	320+++
	Heat	<10	...	6400±	<10
	CHCl₃	1280	...	12,800 Tr.	10
	CHCl₃+heat	320	...	6400	...
T/8/46	...	320	...	3200	160+
	Heat	<10	...	3200	<10
	CHCl₃	1280	51,200	6400	<10
	CHCl₃+heat	320±	51,200	6400	...
T/16/46	...	1280	...	12,800±	10+
	Heat	<10	...	3200	<10
	CHCl₃	2560±	51,200	6400	<10
	CHCl₃+heat	640±	51,200	3200	...
T/Type/J	...	80	...	...	320
	Heat	<20	...	...	<10
	CHCl₃	1280	...	...	<10
	CHCl₃+heat	40	...	...	...

+++ = coarse agglutination by pure Vi antiserum.
+ = fine agglutination by pure Vi antiserum.

* Freshly prepared suspensions from 18-hour agar cultures were centrifuged and suspended in absolute alcohol for 30 minutes to destroy flagellar agglutinability and then, after re-suspension in saline, were divided into three portions. Of these, one was untreated, one was heated at 96° C., the third was treated with chloroform-saline (= CHCl₃); half of this last portion was then heated at 96° C. (= CHCl₃+heat). Parent suspensions were tested for absence of flagellar agglutinability after alcoholisation.

Freshly isolated strains of *Salm. typhi* were similarly tested against normal rabbit sera which showed a wide divergence in titre for T/901/0. untreated and heated.   These sera differed from human serum W.H. in that the sensitivity to them of untreated and chloroform- or HCl-treated suspensions was in no case reduced more than four

times after heating, the fall being only slight with the majority of strains. In other words, results did not differ significantly from those with immune antiserum controls.

A chloroform-treated suspension of one freshly isolated strain (T/2/46) which, after heating, showed a marked drop in agglutinability by human serum W.H., was titrated against this serum after treatment with 5 per cent. HCl, alkali, and absolute, 75 per cent. and 50 per cent. alcohol. The results were similar to those given by T/901/0, treatment with alkali, 75 per cent. and 50 per cent. alcohol producing a marked fall in agglutinability. In order to furnish additional evidence of the similarity of the antigen-antibody system involved in the agglutination of T/901/0 and recent strains, human serum W.H. and normal rabbit serum were absorbed with suspensions of untreated T/901/0 and chloroform-treated T/2/46. Each suspension removed all agglutinin against the other from both sera.

The effect of colony selection on the somatic agglutinability
of Salm. typhi

The considerable differences in relative agglutinability of heated and unheated chloroform-treated suspensions observed for different strains of *Salm. typhi* (table IV) suggested that there might be discontinuous variation in the direction of either stability or lability to heat. Agar-slope suspensions were prepared from 47 colonies of T/901/0 and tested against normal rabbit serum, but no variation towards heat stability could be demonstrated. Two recently isolated strains of *Salm. typhi* were then selected for test—T/80/46, which showed a 16- to 32-fold reduction, and T/16/46, with only a 4-fold reduction in agglutinability by serum W.H. after heating. These were plated, but heated suspensions prepared from 10 colonies of each showed no significant variation from the parent culture. Both

T/80/46.

16 32 16 16 16

T/16/46.

4   4   4   4   4

— 30 serial subcultures on agar—

16 16 16   8 16

4   8 16 16>64
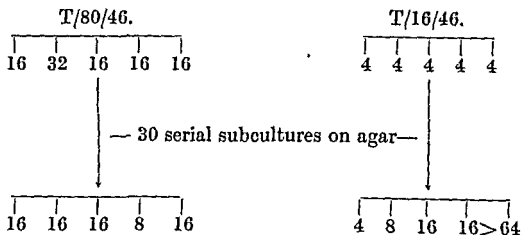
strains were then passaged daily for 30 days on agar slopes. The thirtieth subculture was plated and suspensions made from 5 colonies of each strain. These were tested after chloroform treatment, with and without heating, against human serum W.H. The results may best be indicated by a diagram in which the figures represent, for

each colony, the ratio $\dfrac{\text{titre against heated suspension}}{\text{titre against unheated suspension}}$. Thus a high figure indicates a high degree of heat lability and *vice versa*.

There would appear, therefore, to be a variation in the direction of heat lability which is conditioned by artificial culture.

Complement-fixation tests

It was thought desirable to confirm the loss of reactivity of heated T/901/0 suspension by an antigen-antibody reaction other than agglutination. Complement fixation is the only method whereby the suspensions previously employed could be tested comparatively. Equal volumes (4 standard drops) of serum, antigen, complement and sensitised sheep R.B.C. were used. Saline suspensions of T/901/0 of a density equivalent to 2000 million *Bact. coli* per ml. were found satisfactory as antigen and were not anticomplementary. The opacity of stronger suspensions interfered with the reading of results, and weaker suspensions were less effective in fixing complement. Complement titrations were carried out in the presence of antigen. The antigen-serum-complement mixture was kept for one hour at room temperature before sensitised R.B.C. were added. Results were read after a subsequent $\frac{3}{4}$-hour incubation at 37° C. Allowing fixation to proceed for 20 hours at 4° C. before the addition of sensitised R.B.C. did not markedly increase the complement-fixing titre, which was taken as the highest dilution of serum giving partial fixation.

Using untreated suspension as antigen and slightly less than 2 M.H.D. of complement, the titre of normal rabbit serum was only one-eighth to one-sixteenth, and of human serum W.H. one sixty-fourth that of the agglutinin titre. The use of heated suspension as antigen reduced the complement-fixing titre of the human serum only four times compared with a fall in agglutinin titre of more than 128 times ; with the only rabbit serum tested the titre was halved as against an eight-fold reduction in agglutinin titre. Thus the difference in reactivity between untreated and heated suspensions can be demonstrated by means of complement fixation but this difference is nothing like so marked as in the agglutination reaction.

DISCUSSION

In attempting to find an explanation of the phenomena presented above, several possibilities were considered. The heat lability of T/901/0 and the fact that certain cultures of *Salm. typhi* strain Vi(1) appeared to induce antibody production only against unheated T/901/0 suspension suggested the presence in these two strains of a heat-labile somatic antigen to which antibody occurred in the sera of the majority of normal rabbits and of man. If such an antigen failed to mask the normal O complex, agglutination by high-titre O antisera should not be affected by heating. This has been shown to be

the case. The presence of α-antigen in T/901/0 was excluded, since suspensions of this strain are not agglutinable by α-antisera and do not stimulate α-antibody on inoculation in rabbits. Moreover, there is no correlation between α-titre and that against T/901/0 in a large number of rabbit sera tested. The ability of heated suspension and of inagglutinable strains of *Salm. enteritidis* to absorb antibody against T/901/0 from human and rabbit sera was a point against the heat-labile antigen theory, although it did not exclude it. For example, the agglutinability of Vi antigen and of flagellar antigens in general is usually destroyed by boiling for one or two minutes, but prolonged boiling is required to remove antigenicity and absorbing power. With *Salm. enteritidis* the antigen present could be so arranged spatially in relation to O as to be inagglutinable and yet capable of absorbing. Such conditions exist in fact between mono-specific *Br. abortus* and *melitensis* antisera and suspensions of heterologous type. But the variable behaviour of freshly isolated strains of *Salm. typhi* presented a more convincing case against a heat-labile antigen. After chloroform treatment, all the strains tested have shown approximately the same agglutinability by human and normal rabbit serum as T/901/0. With some strains agglutinability is greatly reduced after heating, with others the sensitivity is little changed, especially to rabbit serum. Both types are capable of absorbing all agglutinin against T/901/0. If, therefore, a heat-labile antigen is present in T/901/0, a more or less heat-stable form of it is also present in freshly isolated strains. Many strains of *Salm. enteritidis*, including both *dublin* and *gaertner* types, have been tested against human and normal rabbit serum. None was agglutinated to a significant titre by human serum W.H. With rabbit sera, some strains were not agglutinated even by a 1 : 10 dilution, while others proved as sensitive as T/901/0. Some strains of intermediate sensitivity were also demonstrated. The agglutinability of these sensitive strains was unaffected by boiling.

It seemed possible that one or more of the normal somatic antigens were subject to a form of variation towards increasing heat lability, without loss of reactivity in the unheated state. Thus all the observed phenomena would be accounted for by the presence in human and normal rabbit serum of antibody against the heat-labile antigen or antigens only, whereas immune sera, which agglutinate both heated and unheated suspensions to about the same titre, contain agglutinins of equal titre for both stable and labile antigens. The somatic antigenic structure of *Salm. typhi* is given by Bornstein as IX, XII, (Vi). Kauffmann has further subdivided the factor XII into three components, XII_1, XII_2 and XII_3, all of which are possessed by strain 901/0 and the great majority of strains of *Salm. typhi*. The component XII_2 undergoes a form of qualitative variation which can be demonstrated in the same way as flagellar diphasic variation by testing colonies with a pure XII_2 antiserum. The culture of T/901/0 used in these experiments is strongly in the XII_2-dominant phase, as

have been all the suspensions prepared from subcultures of it and used latterly in this work. These suspensions, however, are also agglutinable by factor IX and factor XII_3 antisera, though considerably less so than by XII_2 antiserum. In order to determine whether or not any of the known somatic constituents of T/901/0 were heat-labile, pure antisera to factors IX, XII_2 and XII_3 were titrated against untreated and heated suspensions. Heating never reduced agglutinability to less than half. Kauffmann has also confirmed the heat stability of the components of factor XII in strain 901/0. Thus the phenomena shown by human and normal rabbit sera cannot be ascribed to any of these antigens. The demonstration (table III) that human sera fail to agglutinate unheated suspensions of any of the three *Salm. enteritidis* strains to a significantly higher titre than T/901/0 (heated) offers corroborative evidence for the normality of the somatic complex in suspensions of the latter.

The salmonella antigen R has been described as showing a variable decrease in agglutinability without loss of absorbing power on boiling (Savage and White, 1925). In this connection it is interesting to note that Gardner (1928-29) records a 3- to 6-fold drop in the agglutinability by homologous O antiserum of suspensions of his Rawlings strain of *Salm. typhi* after boiling. This was accompanied, however, by an even more marked concomitant rise in agglutinability by " rough " antiserum, which led him to suggest that boiling might unmask a de-stabilising substance in the bacilli, perhaps by the removal of stabilising protein. Although antibody to salmonella R antigen is either not demonstrable or is present only to low titre in rabbit and human serum, it seemed possible that the low apparent titre might be greatly increased, in accordance with the law of optimal proportions, if only a small amount of R antigen were exposed on the surface of T/901/0. Against this were the observations that suspensions prepared from selected smooth colonies showed no sign of saline instability ; that the type of agglutination was characteristically coarse and in no way like the fine muddy agglutination associated with R reactions ; that the antibody concerned was readily removed by smooth suspensions of freshly isolated *Salm. typhi* and *Salm. enteritidis* concerning which there can be no question of roughness ; and that certain cultures of strain Vi(1), which certainly contained R antigen, did not give rise to any increase in titre against T/901/0 on injection into rabbits (table II). It has been recorded above that a suspension of a first generation rough variant of *Salm. enteritidis* strain 12316/JT was able, by absorption, to reduce the titre of human serum W.H. from 1 : 1280 to 1 : 160 but failed to remove antibody from human serum M.B. It also failed to remove significant antibody from two high-titre normal rabbit sera. This rough suspension on injection into a rabbit behaved like strain Vi(1) in that it raised the titre of the rabbit's serum against untreated T/901/0 from $1 : 20 \pm$ to 1 : 320 without affecting that against heated suspension, which

remained stable at 1 : 10. Absorption of this serum with smooth homologous suspension removed all agglutinin against T/901/0 without lowering its titre for the rough variant. The pure R antiserum so produced did not agglutinate either untreated or heated T/901/0. Thus the rough suspension which absorbed agglutinin from human serum W.H. cannot be considered completely rough nor can the agglutination of T/901/0 by this serum be regarded as a rough phenomenon.

It has been pointed out that the titres of *Salm. typhi* and *Salm. enteritidis* O-immune antisera may differ by as much as four times when tested against untreated and heated T/901/0 suspensions. A few *Salm. enteritidis* O antisera have been produced which show no difference in titre as between these same suspensions. Moreover, sera which show a fourfold difference in titre retain this characteristic after absorption with *Salm. enteritidis* strains to produce component-specific XII antisera. Such anomalies of behaviour between different immune antisera and between human and normal rabbit serum in relation to freshly isolated strains suggest that some factor in the serum itself may play a dominant role in the phenomena described. It is possible, for example, that heating may so alter the physical structure of the somatic complex of certain strains of *Salm. typhi* that colloid is non-specifically adsorbed from the serum, protecting the organism against antibody action.

The possibility of inhibition of agglutination resulting from agglutinin deviation by soluble antigenic components liberated from the suspension by boiling (White, 1931) was disproved by the fact that washing suspensions of T/901/0 (heated) in saline did not increase their agglutinability by either human or rabbit sera.

A discussion of the practical implications of these phenomena must await more information about their nature. It is clear, however, that the treatment by heat or alcohol of *Salm. typhi* suspensions for use in the Widal reaction, or even prolonged storage or subjection to temperatures of over 50° C. overnight in the actual test, may profoundly affect the apparent titres given by human sera, even when the sensitivity of such suspensions is found to be normal on testing against rabbit immune antisera.

SUMMARY

1. The serum of normal rabbits agglutinates suspensions of *Salm. typhi* strain 901/0 and of freshly isolated strains after removal of Vi antigen to a markedly higher titre than similarly prepared suspensions of other salmonella types.

2. Both human and normal rabbit serum may show a high titre against suspensions of strain 901/0, but a much lower titre against the same suspension after boiling or treatment with 75 or 50 per cent. alcohol. This phenomenon is absent or apparent only to a slight degree in control experiments with high-titre immune O antiserum.

3. There is an absence of proportional relationship between the titres for untreated and boiled strain 901/0, since the agglutinability of boiled suspensions by normal rabbit sera remains fairly constant whereas that of untreated suspension varies widely.

4. Certain cultures of *Salm. typhi* strain Vi(1) and a partially rough strain of *Salm. enteritidis* have been shown, by rabbit inoculation, to stimulate the production of agglutinins against untreated but not against boiled strain 901/0.

5. Suspensions of freshly isolated strains of *Salm. typhi*, after removal of Vi antigen, showed little loss of agglutinability by normal rabbit serum after boiling, but against human serum some strains showed a fall comparable to that displayed by strain 901/0; others were stable to heat.

6. It is probable that such heat-stable strains undergo a form of discontinuous variation towards heat lability and that this change is induced by artificial culture.

7. Possible explanations of these phenomena are discussed in detail.

REFERENCES

BHATNAGAR, S. S., SPEECHLY, C. G. J., AND SINGH, M. 1938. *J. Hyg.*, xxxviii, 663.

BORNSTEIN, S. 1943. *J. Immunol.*, xlvi, 439.

BRIDGES, R. F. 1943-44. *Trans. Roy. Soc. Trop. Med. & Hyg.*, xxxvii, 343.

FELIX, A. 1924. *J. Immunol.*, ix, 115.

„ 1930. *Lancet*, i, 505.

FELIX, A., BHATNAGAR, S. S., AND PITT, R. M. 1934. *Brit. J. Exp. Path.*, xv, 346.

FELIX, A., AND PITT, R. M. 1934a. This *Journal*, xxxviii, 409.

„ „ „ 1934b. *Lancet*, ii, 186.

„ „ „ 1936. *Brit. J. Exp. Path.*, xvii, 81.

FRANCIS, A. E., AND BUCKLAND, F. E. 1945. *J. Roy. Army Med. Corps*, lxxxiv, 163.

GARDNER, A. D. 1928-29. *J. Hyg.*, xxviii, 376.

KAUFFMANN, F. 1941. *J. Bact.*, xli, 127.

SAVAGE, W. G., AND WHITE, P. B. 1925. Medical Research Council, Spec. Rep. Ser. no. 91, London, pp. 71 and 72.

SCHÜTZE, H. 1930. *Brit. J. Exp. Path.*, xi, 34.

STAMP, (LORD), AND STONE, D. M. 1943-44. *J. Hyg.*, xliii, 266.

TOPLEY, W. W. C., AND WILSON, G. S. 1946. Principles of bacteriology and immunity, 3rd ed., revised by G. S. Wilson and A. A. Miles, London, pp. 277 and 1087.

WHITE, P. B. 1931. This *Journal*, xxxiv, 325.

STAPHYLOCOCCAL HÆMOLYSINS ON SHEEP-BLOOD AGAR WITH EVIDENCE FOR A FOURTH HÆMOLYSIN

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(PLATES XI-XIII)

Staphylococcus aureus is known to produce two hæmolysins : α, which lyses rabbit and sheep red cells and is produced in greatest amount when the culture is incubated in carbon dioxide, and β, which lyses sheep but not rabbit cells and is a " hot-cold " lysin. The existence of a third hæmolysin, similar to α, was suggested by the work of Morgan and Graydon (1936) and of Llewellyn Smith and Price (1938). Although it has been usual to demonstrate hæmolysin production with fluid cultures (*e.g.* McFarlan, 1938 ; Cowan, 1938), several workers have used sheep-blood agar plates for this purpose (Naidu, 1934 ; Bryce and Rountree, 1936 ; Kojima and Kodama, 1939 ; Christie and North, 1941 ; Christie, North and Parkin, 1946) ; none however has reported any extensive investigation of the plate method compared with the tube methods. Because we desired to take advantage of the rapidity of the plate method in epidemiological surveys, we decided to test a number of strains of *Staph. aureus* by the two methods in parallel. However in a preliminary investigation of the plate method we obtained evidence for the existence of a hæmolysin that is apparently distinct from the classical hæmolysins, and since it caused some confusion in the reading of the plates, we made a number of investigations into its nature.

METHODS

Plate tests. In the first place we investigated the conditions in which strains of *Staph. aureus* known to produce α-hæmolysin in fluid cultures gave the widest zones of hæmolysis on blood-agar plates. Either rabbit or sheep blood could be used, but sheep blood gave the more clear-cut results and its use allowed the recognition of β- as well as α-hæmolysin. After most of the preliminary investigations had been completed it was found that the blood supplied for Wassermann tests, containing 0·1 per cent. formaldehyde, was satisfactory for routine use.

The zones of hæmolysis were wider in plates containing 2 per cent. blood than in those with 5 or 10 per cent. (table I), but alterations in the thickness of

the agar, with constant blood concentration, made very little difference. Incubation in air containing added carbon dioxide was found essential for the production of good zones. The plates could be read after 24 hours but the optimum time of incubation appeared to be about 48 hours.

TABLE I

Effect of blood concentration on width of α-hæmolytic zone

Strain no.	Width of hæmolytic zone from colony edge (mm.)		
	2 per cent. sheep blood	5 per cent. sheep blood	10 per cent. sheep blood
1	8·3	5·0	4·5
2	2·3	1·5	1·0
3	11·0	8·5	8·0
4	12·0	10·0	8·0
5	9·0	7·5	6·5
6	9·5	7·5	6·5

Each figure represents the mean of four tests, on different plates. All plates contained 15 c.c. agar and were incubated for 48 hours in air+30 per cent. CO_2.

A plate containing 2·0 units of α-antitoxin per c.c. of medium was always included as a control; the effect of increasing the antitoxin concentration in the control plate is shown in table II. The hazy-edged zone of hæmolysis,

TABLE II

Effect of antitoxin on the hæmolytic zone around colonies of
Staph. aureus

α-antitoxin (units per c.c.)	Width of hæmolytic zone from colony edge (mm.)	Nature of edge of hæmolytic zone
0	5·1	Hazy, due to α-lysin (fig. 1, IC)
0·3	2·9	
0·6	2·2	Clear-cut, due to δ-lysin (fig. 1, ID)
1·2	1·5	
2·4	1·2	
4·8	0·7	

which, as discussed below, seems to be due to the α-hæmolysin, was absent from plates containing as little as 0·3 unit per c.c.; we subsequently adopted 2 units per c.c. for our routine plates as a convenience to reduce the width of the residual clear-cut hæmolysis that is less affected by the antitoxin.

Inoculation was most satisfactorily carried out with a straight wire charged from a colony on a solid medium and stabbed perpendicularly into the agar, 16-20 strains being inoculated on to one four-inch plate. A test showed no detectable difference in the size of the hæmolytic zones produced by 10 colonies arising from 10 consecutive stabs made without recharging the wire, so the test and control plates were routinely inoculated without intermediate recharging.

Tube tests. The tube tests for hæmolysin production were carried out by the method of McFarlan: the cocci were grown for two days in nutrient broth, in air containing 30 per cent. carbon dioxide, and the broth was then tested

against rabbit red cells. A control containing 10 units α-antitoxin per c.c. was included for each strain.

Source of strains. The strains of staphylococci and other organisms were isolated from routine cultures of wounds, noses and skins; they were usually examined within three weeks of isolation, being stored in nutrient broth at 4° C. until tested. Organisms were regarded as being *Staph. aureus* if they gave a good positive reaction with the slide test of Cadness-Graves and co-workers (1943) or with tube tests no. 8 or 9 of Williams and Harper (1946).

RESULTS

Reactions observed on sheep-blood agar plates

Fig. 1 shows the reactions observed with four different strains of *Staph. aureus* producing (I) α-hæmolysin, (II) β-hæmolysin, (III) both α- and β-hæmolysins, and (IV) no detectable hæmolysin when inoculated on sheep-blood agar and incubated as shown in the legend. The reactions in column A resemble those recorded by previous workers, except that the zones of hæmolysis on our aerobic plates were narrower than those illustrated by others. This may have been partly due to the small amount of formaldehyde in the blood, which appears to have a very slight inhibitory effect on the hæmolysis. It may also be that our sheep blood contained more natural antitoxin than theirs, for after completing the greater part of this work we found that wider zones of hæmolysis were produced on plates containing washed sheep cells in place of whole blood (fig. 2), and a test of one sample of the plasma from our blood showed that it contained about 4 units of α-, and 12 units of β-antitoxin per c.c. Munch-Petersen *et al.* (1945) record a similar observation.

The hazy-edged zone of hæmolysis (fig. 1; IC) is that due to the α-hæmolysin. It is neutralised by small amounts of α-antitoxin, its production by different strains runs qualitatively parallel with α-hæmolysin production as determined by the tube test (see below), and it is produced best when the cultures are grown in air containing carbon dioxide. A central zone of clear hæmolysis surrounded by a wide zone of darkening or of partial hæmolysis characterises strains producing β-hæmolysin (fig. 1; IIA, IIIA); this reaction is produced almost equally well in air without and in air with carbon dioxide (*cf.* A and C); the degree of hæmolysis is increased by refrigeration (E and F); and, though not illustrated, the hæmolysis is neutralised by an antiserum containing β-antitoxin. It is clear from fig. 1 (II and III) that there was no notable difference in the plate reactions of strains producing β- or α- and β-hæmolysins.

The plate containing α-antitoxin and incubated in carbon dioxide (ID) shows a narrower zone of hæmolysis than that without antitoxin, and the margin of the zone is sharp rather than hazy. But the hæmolysis is not neutralised by the 2 units of antitoxin per c.c. and indeed it is not always completely neutralised by as much as 128 units per c.c. This residual hæmolysis, which we refer to as "δ"

and attribute, for the sake of convenience and without committing ourselves to any opinion about its real nature, to a " δ-hæmolysin ", is apparently distinct from that due to both α- and β-hæmolysins.

Characteristics of the δ-hæmolysin

The characteristics attributable to the δ-hæmolysin are set out and compared with those of the α- and β-hæmolysins in table III. We observed the δ-hæmolysin effect with all the strains of *Staph. aureus* that produced either α- or β-hæmolysin, but not with any that produced neither of these nor with any coagulase-negative strains.

TABLE III

Comparison of hæmolysis due to α-, β-, and δ-lysins

		α	β	δ
Plate cultures	Demonstrable in air without added CO_2	±	+	—
	Demonstrable in air with 30 per cent. CO_2	+	+	+
	Demonstrable on plates buffered to pH 6 (in air)	±	+	+
	Zone increased by refrigeration after incubation	—	+	—
	Zone reduced by small amounts of α- or αβ-antitoxin (see table II)	+	+	Very slightly
	Demonstrable on plates containing red cells of :—			
	Rabbit	+	—	+
	Sheep	+	+	+
	Man, monkey, horse, rat, mouse, guinea-pig	Trace	—	+
	Effect on specified hæmolytic zone of previous action of β-hæmolysin on the blood agar (see fig. 3)	Decrease	No effect	Increase
Fluid cultures	Hæmolysin demonstrable in broth cultures incubated in air+CO_2	+	+	—
	Hæmolysin demonstrable in semi-solid agar cultures incubated in air+CO_2	+	+	Occasionally

We never demonstrated the presence of a δ-hæmolysin in broth cultures of *Staph. aureus*, even when the cultures were incubated under carbon dioxide or were buffered to pH 6. The lysin was however produced by some strains when grown in broth containing 0·3 per cent. agar, and it seems likely that the anomalous hæmolysin mentioned in a previous paper (Williams and Harper) was in fact the δ-hæmolysin.

The only method that we found consistently satisfactory for obtaining the δ-hæmolysin in a fluid medium was that described by Birch-Hirschfeld (1933-34), namely growing the cocci on the surface of a sheet of cellophane laid on nutrient agar and washing the growth off each plate with about 1·5 c.c. of saline. Twenty strains tested in this way all showed, in the supernatant fluid from the centrifuged

STAPHYLOCOCCAL HÆMOLYSINS
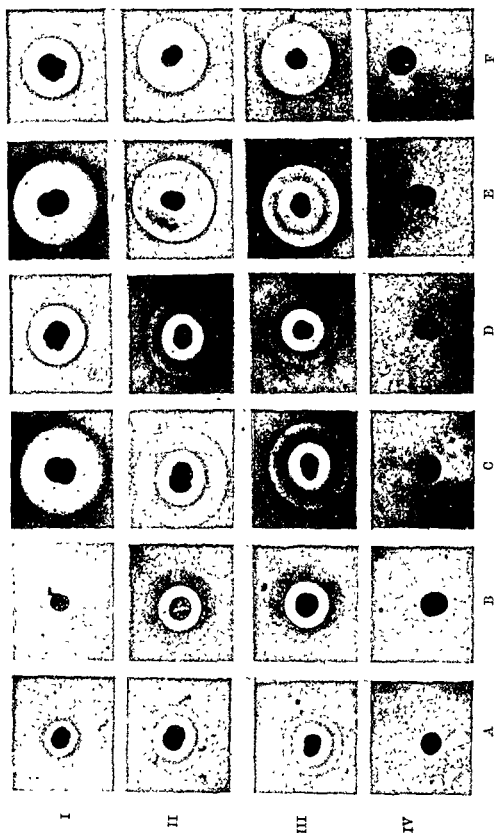
FIG. 1.—Reactions observed on sheep-blood agar plates inoculated with four strains of *Staph. aureus*: (I) α-toxigenic, (II) β-toxigenic, (III) αβ-toxigenic, and (IV) non-toxigenic. The plates in columns A and B were incubated in air, C and D in air with 30 per cent. CO₂, and E and F as C and D but with subsequent refrigeration at 4° C. for 18 hours; plates in columns B, D, and F had 2 units α-antitoxin per c.c. agar. Plates incubated for 48 hours. The apparent effect of the antitoxin in darkening the zone in IIB and IIIB is not constant.

washings, a hæmolysin active against a 1 per cent. suspension of human red cells—a characteristic distinguishing it from either α- or β-hæmolysin—to a titre of at least 1 : 8 and up to 1 : 512 in some cases ; the lysin was not neutralised by 20 units of α-antitoxin per c.c. The cellophane appears to prevent the diffusion of the lysin into the agar for, although δ-hæmolysin activity could be demonstrated from the surface growth of plain agar plates, its titre, in four experiments, was less than that in washings from cellophane-agar plates.

The nature of the δ-hæmolysin

There seems no doubt from the evidence presented that the hæmolysis observed on sheep-blood agar plates containing antitoxin is distinct from that due to the α- and β-hæmolysins ; the wide variety of bloods on which it is active is also evidence against its being a manifestation of the $α_2$- or γ-hæmolysins of Morgan and Graydon and Llewellyn Smith and Price respectively.

In 1941 Christie and Graydon observed that all the strains of *Staph. aureus* that produced hydrolysis of butter fat also produced hæmolysis in areas of sheep-blood agar that had previously been attacked by the staphylococcal β-hæmolysin. We refer to this type of hæmolysis as " β-conditioned hæmolysis ". The hæmolysis illustrated in their paper closely resembles that which we have observed with strains of *Staph. aureus* on antitoxin-containing sheep-blood agar incubated in carbon dioxide ; that is, the hæmolysis we have attributed to the δ-lysin. We thought that the δ-lysin might be the same as the lipolytic enzyme of Christie and Graydon and that the sheep red cells attacked by the β-hæmolysin were, by that attack, made susceptible to the action of the δ-hæmolysin in air, although without this preparation the cells could be lysed only when the plate was made acid or incubated in carbon dioxide. This idea gained strength from the observation that all of 100 unselected strains of *Staph. aureus* were lipolytic on a butter-fat plate, and similarly all of a number of strains of *Staph. aureus* tested have shown both the β-conditioned lysis on aerobic plates and potentiation of the δ-lysis by β-hæmolysin on antitoxin plates incubated in carbon dioxide.

There are however a number of observations suggesting that the δ-hæmolysin in not the same as the lipase. Thus we were able to separate the δ-hæmolysin from the lipase by the method described by Birch-Hirschfeld (1936-37) : supernatants from cultures of *Staph. aureus* grown on cellophane on agar were precipitated with alcohol and ether, and the precipitate redissolved in saline. This saline solution had no action on human cells, but still contained a lysin active against rabbit cells and neutralised by α-antitoxin (table IV). The presence of lipase was demonstrated by putting a few drops of the fluid, to which penicillin had been added to prevent the growth of

staphylococci, into penicillin-assay cylinders on agar plates containing 0·1 per cent. washed butter fat and 0·005 per cent. neutral red. The esterase was present in almost equal titre before and after precipita-

TABLE IV

Separation of δ- from α-hæmolysin by alcohol and ether precipitation

	Original extract	Extract after precipitation and redissolving
Titre for rabbit cells in tube	1 : 128	1 : 64
Titre for human cells in tube	1 : 32	0
Titre for sheep cells in tube	1 : 8 Tr.	1 : 2
Titre for human cells in plate *	1 : 32	0
Esterase titre on butter-fat plate *	1 : 64	1 : 64
Units per c.c. staphylococcal α-antiserum required to neutralise hæmolysis of :—		
Rabbit cells	>320	<10
Human cells	>320	<10
Sheep cells	Tr. in all, i.e. >320	<10

Tr. = trace.

* Extracts tested in penicillin-assay cylinders on 2 per cent. human red cell, or 0·1 per cent. butter-fat plates respectively.

tion. Birch-Hirschfeld described the separation by this precipitation of the tributyrin esterase from the "hæmolysin" but her method of estimating hæmolysin would detect α-, β-, or δ-hæmolysin and it may be that the reduced titre that she observed was due to a reduction in the δ-hæmolysin titre.

Furthermore the phenomenon of β-conditioned hæmolysis is not one manifested only by *Staph. aureus.* Christie and his colleagues (Christie and Graydon, 1941 ; Christie *et al.*, 1944 ; Munch-Petersen *et al.*, 1945) demonstrated the phenomenon with all strains of group-B streptococci and with some "air-borne" micrococci and contaminants. We too have observed it with micrococci, as well as with an aerobic spore-bearing organism and an unidentified mould isolated as contaminants on routine sheep-blood plates ; none of these organisms produced hæmolysis of the δ type on sheep-blood plates incubated in carbon dioxide. And although all strains of *Staph. aureus* that showed β-conditioned hæmolysis also showed hydrolysis on a butter-fat plate, this was not true of other organisms. Of 20 coagulase-negative staphylococci, we found 13 which showed both β-conditioned hæmolysis and a butter-fat esterase, one which showed the former only, *four* which showed the latter and two which showed neither. The group-B streptococci were not lipolytic, nor was the mould or the spore-bearer. The lack of association between lipase production and ability to produce β-conditioned hæmolysis seems against the view that the lipase is responsible for the hæmolysis.

PLATE XII

STAPHYLOCOCCAL HÆMOLYSINS
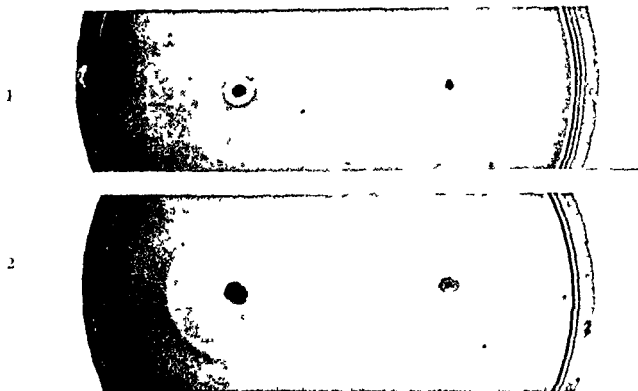
FIG 2 —Zones of hæmolysis produced by α and β toxigenic strains of *Staph aureus* on plates containing (1) whole sheep blood, and (2) washed sheep red cells   Plates incubated 48 hours in air with 30 per cent CO₂.
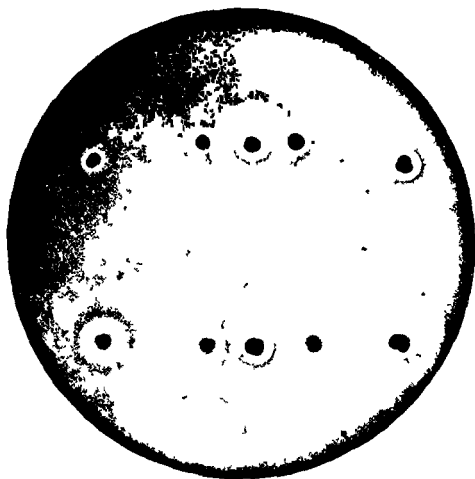
FIG 3 —Potentiation of δ hæmolysin by β hæmolysin   Central colonies are those of a β toxigenic strain   others of α toxigenic strains   Plate incubated in air with 30 per cent CO₂ for 48 hours , α antitoxin spread over top half of plate before inoculation

.

The fact that many organisms produce β-conditioned hæmolysis but no δ-hæmolysin active against human red cells or demonstrable on a sheep-blood plate incubated in carbon dioxide suggests either that the δ-hæmolysin is not responsible for the β-conditioned hæmolysis produced by *Staph. aureus* or that such hæmolysis can be produced by a variety of agents. Tentatively, we suggest that the β-hæmolysin acts on the sheep cells in some way so as to increase their susceptibility to lysis by : (1) cold, (2) some agent or agents in cultures of *Staph. aureus*, group-B streptococci, etc., growing aerobically, and (3) the δ-hæmolysin produced by *Staph. aureus* grown in carbon dioxide.

In connection with β-conditioned hæmolysis there are two further points of interest. Firstly, as shown in fig. 1, β-toxigenic strains of staphylococci produce a clear zone of hæmolysis central to the darkening due to the hot-cold lysin. The clear zone was thought by Bryce and Rountree to be due to the α-hæmolysin, but we have found it equally obvious with strains producing no demonstrable α-hæmolysin in tube tests, and we consider it another example of β-conditioned hæmolysis, both the conditioning and the hæmolytic agent being produced by the same colony. Secondly, four strains of *Cl. welchii* stabbed into sheep-blood plates and incubated anaerobically produced a zone of darkening which became clear on refrigeration, just like that produced by the staphylococcal β-hæmolysin ; this " hot-cold " lysis was shown by van Heyningen (1941) to be due to the *Cl. welchii* α-toxin. Staphylococci stabbed into the zone of darkening produced hæmolysis of the " β-conditioned " type (fig. 4). There was no cross-neutralisation of the *Cl. welchii* hæmolysis by the staphylococcal β-antitoxin, or conversely.

The use of sheep-blood agar plates

Comparison of plate and tube methods for detecting hæmolysin production. Using the criteria for the diagnosis of α- and β-hæmolysins discussed above, and requiring that any hæmolysis on the control plate should be of the sharply defined δ-variety, we tested 287 strains of coagulase-positive staphylococci (237 toxigenic and 50 non-toxigenic) in parallel by the plate and tube methods. There was complete agreement in the results. In the course of routine investigations 2267 strains of *Staph. aureus* (some being repeat strains from one wound) were tested on plates ; of these, 2108 were regarded as producing α-hæmolysin, 42 as producing β-hæmolysin and 117 as producing neither hæmolysin. Since the β-hæmolysin masks the α on the plate, tube tests were performed on the 42 strains showing β-hæmolysin ; 29 (69 per cent.) produced α-hæmolysin as well. Of the 117 strains producing no hæmolysis on the plate, 27 (23 per cent.) produced some α-hæmolysin in a tube test, although in 19 the hæmolysis was weak. The plate test therefore missed 56 of the 2164 α-toxigenic strains, 27 being false negatives on the plate and 29

β-toxin producers and therefore unrecognisable as α-toxin producers on the plate. If washed sheep cells are used instead of whole blood, up to 10 per cent. of strains can be shown to produce some β-hæmolysin, and a greater proportion have therefore to be tested in tubes to determine α-hæmolysin production.

We also tested on the plates 152 strains of coagulase-negative staphylococci and micrococci, 15 strains of coliform and 11 of diphtheroid organisms, and nine strains of *Str. pyogenes*. None gave a positive result as we have defined it.

Sheep-blood agar plate for rapid recognition of Staph. aureus. The sheep-blood agar plate may also be used for the detection of *Staph. aureus* in mixed cultures in the way suggested by Penfold (1944) and Reid and Jackson (1945) for the plasma-agar plate. Fig. 5 shows a sheep-blood agar plate, inoculated from a swab containing *Staph. aureus* and micrococci, after overnight incubation in air containing 30 per cent. carbon dioxide; two drops of staphylococcal antiserum had been spread over half the plate before inoculation. The colonies of toxigenic *Staph. aureus* are readily distinguishable on the test half by the hæmolysis, and this zone of hæmolysis is far easier to recognise than the halo on the plasma-agar plate. Furthermore only about 0·25 c.c. of sheep blood is required for each plate, compared with about 2·5 c.c. of a specially selected sample of human plasma for the plasma-agar plate.

DISCUSSION

We had some evidence from previous work (Schwabacher *et al.*, 1945) that α-toxigenic strains of *Staph. aureus* were more pathogenic for wounds than non-toxigenic strains, and we wished to include determinations of toxin production in surveys of staphylococci isolated from wounded patients. The number of strains to be tested would have made the use of a tube technique extremely laborious, and accordingly we decided to test the reliability of the sheep-blood agar plate. Preliminary experiments showed that, in our hands, no more than a narrow zone of hæmolysis was produced when the plates were incubated aerobically, and in view of the recognised importance of carbon dioxide in the production of α-hæmolysin in fluid cultures, we incubated our plates in air containing carbon dioxide. An antitoxin-containing plate was always inoculated in parallel as a control. We can find no record in the literature of the systematic use either of an antitoxin control or of incubation of the plates in carbon dioxide, although Rountree (1936) used such enrichment in her studies of α-β variation, and Rigdon (1938-39) made some observations with antitoxin plates incubated aerobically. These two omissions led to the failure to recognise the hæmolysis that we have referred to as " δ ".

As a method of identifying α-toxigenic *Staph. aureus*, the plate test is confusing at first because of the δ-type of hæmolysis on the antitoxin-containing control plate, but with a little experience it is

STAPHYLOCOCCAL HÆMOLYSINS
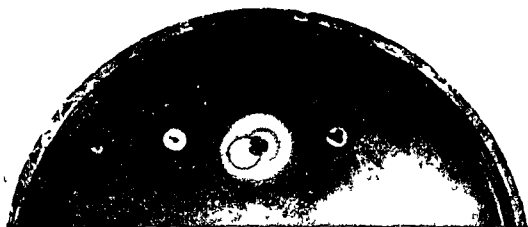
FIG. 4.—Hæmolysis of sheep blood agar previously attacked by *Cl. welchii* toxin. Central colony is *Cl. welchii* (with gas bubble in depth of agar); on the left are two colonies of a group B streptococcus, and on the right two colonies of *Staph. aureus*. The cocci produced hæmolysis only when the cells had been attacked by the *Cl. welchii* toxin, as shown by the darkening. Plate incubated anaerobically for 3 days with *Cl. welchii*, and then aerobically for 18 hours after inoculation with the cocci.
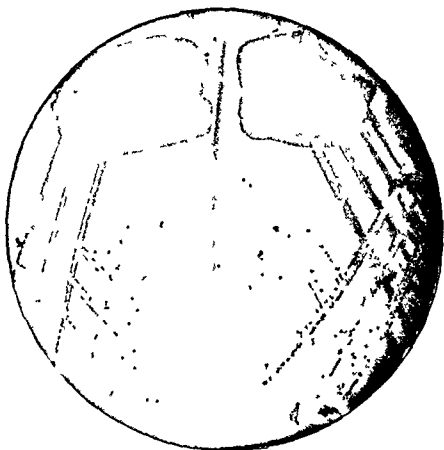
FIG. 5.—Recognition of *Staph. aureus* in mixed culture with micrococci. Plate incubated 24 hours in air with 30 per cent. CO_2; α antitoxin spread over left half of plate before inoculation.

easy to distinguish the two types of hæmolysis and to recognise the α-toxigenic strains. The fact that the plate method fails to demonstrate about 2 per cent. of the α-toxigenic strains, while the β-hæmolysin masks the α on the plate, means that all apparently negative and all evidently β-toxigenic strains must be tested in a tube before being classed as non-α-toxigenic.

SUMMARY

Nutrient agar plates containing 2 per cent. sheep blood, incubated in air containing 30 per cent. carbon dioxide, were found reliable for the detection of hæmolysin production by *Staph. aureus*. Of over 2000 strains tested, 117 produced no hæmolysin on the plates and only 27 of these yielded any hæmolysin in tube tests. It was not found possible to distinguish on the plate the strains that produced β-toxin alone from those producing both α and β.

The plates could also be used for the rapid detection of toxigenic *Staph. aureus* in mixed cultures.

On sheep-blood plates containing α- or αβ-antitoxin, a type of hæmolysis was produced that is distinct from that due to the α- or β-hæmolysin. It was produced only when the plates were incubated in air containing carbon dioxide, but it was equally active on a variety of mammalian bloods, including human. We suggest, tentatively, that it should be referred to as the δ-hæmolysin.

REFERENCES

BIRCH-HIRSCHFELD, L.	. . .	1933-34.	*Z. Immunitätsforsch.*, lxxxi, 260.
„		1936-37.	*Z. Hyg.*, cxix, 431.
BRYCE, L. M., AND ROUNTREE, P. M.		1936.	This *Journal*, xliii, 173.
CADNESS-GRAVES, B., WILLIAMS, R., HARPER, G. J., AND MILES, A. A.		1943.	*Lancet*, i, 736.
CHRISTIE, R., AND GRAYDON, J. J.		1941.	*Austral. J. Exp. Biol. and Med. Sci.*, xix, 9.
CHRISTIE, R., AND NORTH, E. A. .		1941.	*Ibid.*, xix, 323.
CHRISTIE, R., ATKINS, N. E., AND MUNCH-PETERSEN, E.		1944.	*Ibid.*, xxii, 197.
CHRISTIE, R., NORTH, E. A., AND PARKIN, B. J.		1946.	*Ibid.*, xxiv, 73.
COWAN, S. T.		1938.	This *Journal*, xlvi, 31.
VAN HEYNINGEN, W. E. . . .		1941.	*Biochem. J.*, xxxv, 1257.
KOJIMA, T., AND KODAMA, T. .		1939.	*Kitasato Arch. Exp. Med.*, xvi, 197.
McFARLAN, A. M.		1938.	*Brit. Med. J.*, ii, 939.
MORGAN, F. G., AND GRAYDON, J. J.		1936.	This *Journal*, xliii, 385.

MUNCH-PETERSEN, E., CHRISTIE, R., AND SIMMONS, R. T.   1945.   *Austral. J. Exp. Biol. and Med. Sci.,* xxiii, 193.

NAIDU, P. M. N. . . . . .   1934.   *Zbl. Bakt.,* Abt. I, Orig., cxxxii, 47.

PENFOLD, J. B. . . . . . .   1944.   This *Journal,* lvi, 247.

REID, J. D., AND JACKSON, R. M.   1945.   *J. Lab. and Clin. Med.,* xxx, 155.

RIGDON, R. H. . . . . . .   1938-39. *Ibid.,* xxiv, 1264.

ROUNTREE, P. M. . . . . .   1936.   *Austral. J. Exp. Biol. and Med. Sci.,* xiv, 93.

SCHWABACHER, H., CUNLIFFE, A. C., WILLIAMS, R. E. O., AND HARPER, G. J.   1945.   *Brit. J. Exp. Path.,* xxvi, 124.

SMITH, M. LLEWELLYN, AND PRICE, S. A.   1938.   This *Journal,* xlvii, 379.

WILLIAMS, R. E. O., AND HARPER, G. J.   1946.   *Brit. J. Exp. Path.,* xxvii, 72.

THE EFFECTS OF TEMPORARY OCCLUSION OF THE RENAL ARTERY IN RABBITS AND ITS RELATIONSHIP TO TRAUMATIC URÆMIA

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(PLATES XIV-XVII)

SINCE the classical description of crush anuria (Bywaters and Beall, 1941; Beall *et al.*, 1941) increasing interest has been focussed upon the uræmic syndrome which follows severe injury, incompatible blood transfusion, blackwater fever, etc. This syndrome has been largely attributed to a nephrotoxin arising from damaged muscle or hæmolysed red blood cells (Bywaters and Dible, 1942). More recently it has been suggested that the primary ætiological factor may be renal anoxia caused by diminution of blood supply to the kidney (Tomb, 1942; Maegraith and Findlay, 1944; Darmady *et al.*, 1944). Some confirmation of this view was given by Cournand *et al.* (1943) and Lauson *et al.* (1944), who demonstrated that in shock there is a marked alteration in renal blood flow, not to be accounted for by changes in blood pressure or pulse rate; they attributed the change to spasm of vessels. It was thought, therefore, that temporary occlusion of the renal artery might throw some light on this as a possible mechanism.

REVIEW OF THE LITERATURE

Experiments on partial interference with the blood supply to the kidney may be subdivided into (1) those in which the veins were occluded, (2) those in which both artery and vein were clamped or ligatured, and (3) those in which the arterial supply alone was obstructed.

In the first group—venous occlusion—the early work appears to have been concerned with the production of chronic passive congestion. Thus Robinson (1843, quoted by Rowntree *et al.*, 1913) produced enlargement of the kidney, followed by casts and blood in the urine. This work appears to be confirmed by the majority of workers mentioned by Rowntree *et al.* Paneth (1886, quoted by Rowntree *et al.*) seems to have been the first to establish the fact that obstruction of the venous return is followed by oliguria. Similar findings are reported by de Souza (1900-01) and Ignatowsky (1905). These workers were concerned only with keeping their animals alive for a short time—up to four days—and it was left to Rowntree *et al.* to try the effects when life was prolonged. They placed constricting bands either on both renal veins, or on one renal vein after the other kidney had been removed. They conclude that no permanent damage is produced by partial occlusion of one vein, but when both are

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NAIDU, P. M. N. — 1934. *Zbl. Bakt.*, Abt. I, Orig., cxxxii, 47.

PENFOLD, J. B. — 1944. This *Journal*, lvi, 247.

REID, J. D., AND JACKSON, R. M. — 1945. *J. Lab. and Clin. Med.*, xxx, 155.

RIGDON, R. H. — 1938-39. *Ibid.*, xxiv, 1264.

ROUNTREE, P. M. — 1936. *Austral. J. Exp. Biol. and Med. Sci.*, xiv, 93.

SCHWABACHER, H., CUNLIFFE, A. C., WILLIAMS, R. E. O., AND HARPER, G. J. — 1945. *Brit. J. Exp. Path.*, xxvi, 124.

SMITH, M. LLEWELLYN, AND PRICE, S. A. — 1938. This *Journal*, xlvii, 379.

WILLIAMS, R. E. O., AND HARPER, G. J. — 1946. *Brit. J. Exp. Path.*, xxvii, 72.

The remainder of the work on clamping of the renal arteries has been done with the object of producing hypertension, and is therefore not always relevant to the present study. Some of these workers, however, comment on the fact that partial clamping of renal arteries in dogs and rats results in death in uræmia accompanied by excessively raised blood urea (Goldblatt, Lynch *et al.*, 1934; Goldblatt 1937-38; Wilson and Byrom, 1939; Goldblatt, Weinstein and Kahn, 1941). Pickering and Prinzmetal (1937-38), working on the same subject and discussing the relationship of renal blood flow and renal ischæmia, state that partial compression will lead to death in some animals 50-60 hours after operation. At autopsy the kidney is enlarged and soft. Section reveals patchy necrosis of kidney cells and many hæmorrhages are seen. The tubules and glomeruli are distended with albuminous fluid. In other areas the kidney cells appear to be intact.

Maegraith and Maclean (1942), in carrying out clamping experiments in animals, showed that in many there were extensive degenerative changes in the renal tubular epithelium, some distension of tubules and often casts. Verney and Vogt (1943-44) studied the effects of temporary clamping for 2-60 seconds. In some animals they found hypertension and anuria even after this short period of clamping, and suggest that this may be due to continued spasm of the renal artery.

Phillips *et al.* (1945-46), working with dogs, found that when shock was produced by bleeding or trauma the blood flow through the kidneys progressively decreased, and that when a certain degree of shock was reached—attained by loss of 40-45 c.c. of blood per kilo—both renal blood flow and the fraction of water filtered in the glomeruli fell to almost zero levels. In other experiments by the same investigators (Van Slyke *et al.*, 1944), one kidney of each dog was removed and the flow of blood through the other was stopped for varying periods by clamping the renal artery. It was found that the duration of renal ischæmia thus produced bore a direct relation to mortality rate. Thus dogs in which ischæmia was maintained for three hours were all capable of recovering, although there was urea retention for several days. Of those clamped for four hours, 50 per cent. died in uræmia 4-8 days later, whereas no dog was capable of withstanding ischæmia for six hours. Histologically there was damage to the distal tubules but the glomeruli were unaffected.

EXPERIMENTAL METHODS

General procedure and dietetic measures. The rabbits used in these experiments were obtained partly by home breeding and partly from the animal farm of the Experimental Station, Porton. Their weight varied between 1·8 and 3·2 kg. Young adults were used as far as possible. In order to ensure an alkaline urine a carefully controlled diet consisting of green vegetables five days a week and root vegetables on the remaining two days was given. This was supplemented by oats and bran (Yorke and Nauss, 1911-12; Baker and Dodds, 1925). The maintenance of alkaline urine throughout the experiment was considered desirable, since it has been suggested that acidosis might be one of the chief ætiological factors, and also since Bywaters (1945) has suggested that early correction of the acidosis is the underlying principle in treatment. On the day of operation the rabbit was starved and only water given. Following operation a free supply of water and foodstuff was made available, but there was no supplementary administration of water, as by stomach tube.

Operative procedure. The animals were anæsthetised, during operation, with a mixture of ether (80 per cent.) and absolute alcohol (20 per cent.). The operative procedure, carried out by A. W. B., consisted in exposing each kidney separately through an incision in the lumbar dorsal aponeurosis. Care was taken not to damage or open the peritoneum, which was stripped off the front of the kidney with the tip of the index finger. The left kidney was delivered

first and a careful dissection made to separate the artery and vein. When the artery had been freed, a clamp similar to that described by Pickering and Prinzmetal was introduced over the artery only. The clamp was constructed so that the distance between the two limbs was approximately 0·5-0·6 mm. The distal ends of the clamp were tied together, and the renal artery slid into the " V " of the clamp until a " snug " fit was obtained. The kidney, artery and clamp were then returned to the normal renal position. The lumbar dorsal aponeurosis was secured with a temporary stitch and the skin edge clipped. It was hoped by this means to produce a standard degree of compression. Through a new incision the right kidney was similarly exposed, and removed, the lumbar dorsal aponeurosis securely sutured by interrupted thread stitches and the skin edges approximated by clips. The nephrectomy served a three-fold purpose. It allowed the right kidney to be used as a histological control, it obviated the difficulty of maintaining two clamps in position and, since the right kidney is higher and the renal artery shorter and more difficult to isolate, it shortened the length of the operation and diminished " shock ". Following the operation, the animals were allowed to recover consciousness. The clamps were left in position for periods ranging from 1 to 2 hours, care being taken that the variation in the length of clamping was not always done on the same day. At the end of the required period of clamping, the animal was re-anæsthetised with the same mixture, the clamp removed and the wound securely closed.

Biochemical investigations. Although no attempt was made to measure urinary excretion against water ingestion, the urine was tested with litmus paper to confirm that the reaction was alkaline. The degree of urea retention was assessed by the blood-urea level, since this, in human cases of traumatic uræmia, shows the most marked variation. It was estimated before operation

TABLE I

Serial blood-urea levels (mg. per 100 c.c.) in ten control animals
(nephrectomy without clamping)

Rabbit no.	Weight in g.		Blood-urea levels								Remarks
	Initial	Final	Pre-operative	Days after operation							
				2	5	7	12	15	19	28	
41	2808	2892	50	54	55	30	33	41	80	52	With clamps
42	2706	2790	50	64	46	48	33	46	66	46	
44	2780	2848	86	56	40	26	28	51	62	52	
45	2808	2864	88	64	30	27	34	47	98	56	
46	3116	3134	60	122	30	33	50	53	64	...	
61	3144	3280	41	64	53	34	44	...	40	31	Without clamps
62	2836	3032	98	80	56	52	52	...	40	44	
63	3206	3318	48	76	88	48	42	...	40	38	
64	3032	3016	46	88	60	34	41	...	31	42	
65	2920	3060	42	64	68	35	54	...	36	62	

and at varying intervals post-operatively. In view of the work of Meyler (1935) in guinea-pigs and Corcoran and Page (1943) in dogs, in which uræmia was induced by repeated bleeding, it was thought inadvisable to bleed the animals frequently. The quantity of blood used was limited to 0·2 ml. and was obtained from the marginal ear vein. The estimation was carried out according to the technique of Archer and Robb, using an Ogal colorimeter.

Description of the experiment. The rabbits were divided into four groups of ten. The first series was kept as a control. In it the same operative procedure

and double anæsthetic were used, except that in five animals a clamp was introduced but not closed over the artery, while in the remaining five a clamp was not even introduced. In each case right nephrectomy was performed. This gave some indication of the rise in blood urea following the operative procedure, including nephrectomy. *All these animals survived for 28 days and were subsequently killed*; the rise in blood urea did not exceed 120 mg. per 100 c.c. (table I).

In the remaining three series the renal arteries were clamped for periods of 60, 90 and 120 minutes.

RESULTS

These are summarised in table II, which shows that the mortality rate is directly proportional to the period of clamping, being *100 per cent.* in animals whose renal arteries were clamped for 120 minutes,

TABLE II

Experimental series

Survival period (days)	Control	Period of clamping (minutes)			Number showing same renal changes	Renal histology grade
		60	90	120		
2	0	0 ⎫	2 ⎫	4 ⎫	6	I
3–6	0	1 ⎬ 20 per	2 ⎬ 60 per	5 ⎬ 100 per	8	II
7–15	0	1 ⎭ cent.	2 ⎭ cent.	1 ⎭ cent.	4	III
28	10	8	4	0	12	IV
Number of rabbits used	10	10	10	10	...	...

as compared with 60 per cent. and 20 per cent. in those which were clamped for 90 and 60 minutes respectively. Naturally the period of survival shows the converse relationship. Of animals whose arteries were clamped for 120 minutes, 40 per cent. died within two days and another 50 per cent. within seven days, leaving only 10 per cent. which were able to survive into the second week. Of those in which the arteries were clamped for 90 minutes, only 20 per cent. died within two days, 20 per cent. within three to seven days, and 20 per cent. between seven and fifteen days. Both the animals which died after clamping for 60 minutes were able to survive more than five days.

Results of the biochemical investigation

Before passing to the study of the histology of these animals, consideration of the effects of temporary clamping on urea retention is essential. Table I shows that in the controls a rise exceeding 120 mg. per 100 c.c. is unlikely. It is of interest to note that in the animal showing the greatest rise in blood urea a clamp was introduced but not closed. It is possible that during the return of the kidney to the body cavity kinking of the artery around the clamp occurred, with the resulting abnormal rise.

Tables III-V show the serial blood-urea levels in animals of similar survival period and, as will be shown, approximately the same renal histology, four grades of which are recognised. Those surviving less than two days all showed an immediate rise of blood urea (table III).

TABLE III

Blood-urea levels (mg. per 100 c.c.) in rabbits surviving for less than two days after occlusion of the left renal artery and right nephrectomy. (Renal histology, grade I)

Rabbit no.	Initial weight in g.	Blood-urea levels			Period of clamping (mins.)
		Pre-operative	Days after operation		
			1	2	
2	2864	63	188	276	90
5	3032	57	144	176	90
8	3234	70	...	280	120
10	1872	52	...	148	120
13	2566	52	...	168	120
14	2510	52	...	212	120

Animals dying in from three to seven days showed a more gradual rise in blood urea (table IV), although two rose to quite abnormal levels, namely rabbits 7 (700 mg. per 100 c.c.) and 68 (1160 mg. per 100 c.c.).

TABLE IV

Serial blood-urea levels (mg. per 100 c.c.) in rabbits surviving for 3-7 days after occlusion of the left renal artery and right nephrectomy. (Renal histology, grade II)

Rabbit no.	Initial weight in g.	Blood-urea levels					Period of clamping (mins.)
		Pre-operative	Days after operation				
			2	3	4	5	
7	2920	60	432	...	700	Died	120
9	2082	52	136	...	528	...	120
11	1900	46	160	...	424 Died	...	120
12	2622	44	184	228 Died	...	...	120
17	2566	66	208	...	528	Died	90
22	2808	72	142	...	Died	...	90
47	3116	34	...	...	328 Died	...	120
68	3346	53	164	...	1160 Died	...	60
			2	3	4	6	7	
48	3262	30	...	400	...	450	Died	90
49	3178	33	...	300	Died	...	...	90

Rabbits 48 and 49 are not included in table II.

Four rabbits which survived for 7-15 days (nos. 6, 21, 26 and 33) and in which the kidneys showed the histological appearances classed as grade III are of special interest. The blood-urea levels are charted (fig. 1) for comparison with a similar chart (fig. 2) prepared from four human cases of traumatic uræmia reported elsewhere (Darmady et al., 1944, case 4; Darmady, 1946-47, cases 9, 10 and 14). Both show a gradual rise in blood urea over the course of a few days until
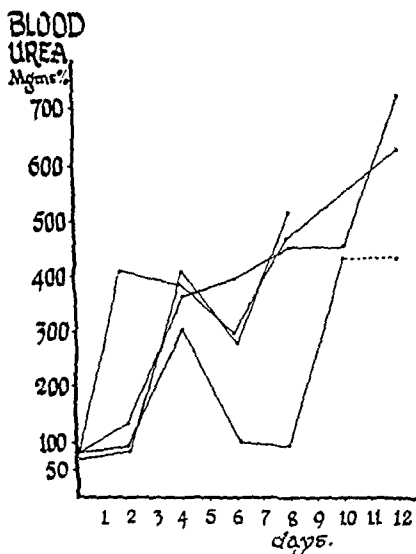
Fig. 1.—*Serial blood-urea estimations in four rabbits with renal histology grade III, showing the rise in the blood urea of rabbits 6, 21, 26 and 33, which were able to survive 7-14 days after temporary occlusion of the renal artery in the unilateral nephrectomised animal. The ability to survive for a period of days at abnormal levels is notable.*

the level reached is quite abnormal (200 mg. per 100 c.c. or more), and continued survival for several more days, even with the blood urea at this level.

Table V shows the serial blood-urea levels in 12 animals which survived clamping for 28 days and were then killed. The particular features of this group are only obvious when compared with those in the control series (table II).

Although urea retention is not perhaps so marked as in the three previous experimental groups, it is certainly greater than in the control group. A point of interest is the secondary rise which occurs
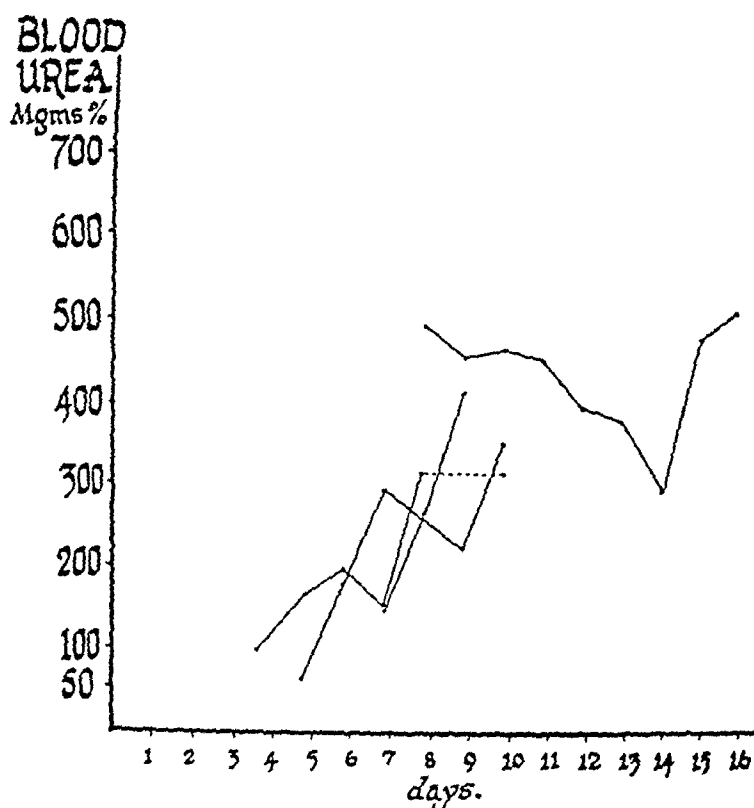
FIG. 2.—Serial blood-urea estimations in four fatal (human) cases of traumatic uræmia. The abnormal levels and the ability of the body to withstand these levels are similar to those shown in fig. 1.

TABLE V

Serial blood-urea levels (mg. per 100 c.c.) in rabbits killed on the 28th day after clamping of the left renal artery and right nephrectomy. (Renal histology, grade IV)

Rabbit no.	Weight (g.)		Blood-urea levels												Period of clamping (mins.)
			Pre-operative	Days after operation											
	Initial	Final		1	2	4	6	8	10	12	14	18	21	28	
1	2948	3116	51	148	...	196	...	...	38	...	...	...	...	43	90
15	2752	2864	64	...	160	232	104	...	54	...	...	31	...	41	90
23	2566	2760	76	...	128	204	312	92	54	...	...	60	33	45	90
24	2510	2678	70	...	136	44	68	156	144	60	48	43	...	40	90
27	2706	2950	48	...	176	106	74	70	96	35	60	...	36	34	60
29	2622	2706	71	...	116	88	80	78	122	38	28	...	37	52	60
30	2566	2734	79	...	136	62	60	60	105	50	27	38	53	60	60
28	2622	2650	60	...	58	48	42	78	122	52	44	...	44	60	60
31	2678	2748	49	...	244	48	46	58	98	50	24	38	59	94	60
32	2724	2808	78	...	384	232	70	115	108	35	...	29	37	60	60
67	3178	3232	47	...	212	480	94	63	47	...	28	...	...	32	60
81	3234	3206	22	...	132	132	58	...	...	49	...	...	...	...	60

in some of the animals about the tenth day. A similar phenomenon is seen in the experimental group which showed grade III renal histology (fig. 1). The significance of this secondary rise is not understood. The rise in the blood urea suggests that interference with the renal circulation has been achieved.

Histology

Routine examination was made of the liver and both kidneys in all animals. In rabbits numbers 1, 2, 5-9, 21, 26 and 33 the suprarenals, lungs and skeletal muscles were also examined, but no abnormal changes were found. In each animal the right kidney was fixed as soon as possible after removal, tangential section in two or more places being made with a sharp razor. Post-mortem examinations were carried out as soon as possible after death. In the main, preservation of organs was good. In two (nos. 11 and 12), where death had occurred during the night, the examination was carried out in the morning and, as the animal house was heated, some degree of post-mortem change could not be avoided. Organs from these animals were not included in the histological study.

In general, post-mortem examination did not reveal any gross abnormality, but œdema of the lungs was sometimes observed and most animals dying within 1 week appeared to have lost weight. Local inflammatory changes were minimal and the wound invariably healed by first intention. The remaining kidney appeared to be rather swollen and the capsule under tension. This was confirmed after section with a sharp razor, when the cortex was seen to bulge and the cut edge was everted. The surface was paler than usual and had a glistening yellow granular appearance. Occasionally, and most markedly in those dying early, frank infarction of some areas of the kidney was seen.

Fixation in each case was carried out in mercuric-formol-saline. As routine, hæmatoxylin and eosin and Masson's trichrome stain were used, and in selected cases van Gieson's stain and Leperhen's stain for hæmoglobin. Staining for free iron was also performed and in some cases frozen sections were stained for fat.

Abnormal changes were found only in the kidney under experiment. In the liver, the central vein necrosis observed in man was not present, nor was there noticeable fatty change. As previously stated, the renal changes fall into four grades, corresponding approximately to the survival periods. It is important to realise that the nephron of the rabbit differs in many ways from that of man (Dunn and Polson, 1926). The ascending and descending limbs in the rabbit may descend into the pyramids and loop round the collecting tubules, other nephrons descend to the boundary zone only. Interest centres chiefly on the lowest part of the loops of Henle.

Renal histology, grade I. This type of histology was seen in six animals which died within two days. One of the principal features is the comparatively normal appearance of the glomeruli, although in

certain cases there is some distortion of the tuft. There is also albuminous deposit in the capsular space. The most striking changes are the almost complete destruction and degeneration of the first and second convoluted tubules. Nuclear staining has disappeared and the epithelium is replaced by a thin rim of eosinophilic material (fig. 3). No cast formation is seen in the second convoluted tubules. On examining the nephron at a lower level in the region of the pyramid, there is some degeneration of the epithelium and some cast formation. The collecting tubules show degeneration of the epithelium with some minimal cast formation. These various changes are similar to those described by Scarff and Keele, and are comparable in some respects to those of human cortical necrosis due to vascular stasis.

Renal histology, grade II. Eight animals showed changes which belong to this grade. The principal feature here is the patchy necrosis found in the cortex (fig. 4). Some areas show frank necrosis resembling that seen in grade I ; others—the more viable portions—show changes similar to those of grade III. The demarcation between these areas is frequently clear-cut. In support of the view that the epithelium is not entirely functionless is the presence of casts in the second convoluted tubules. A further abnormal change is the dilatation of the secreting tubules, more often seen in the cortex (fig. 5). This change is perhaps a little difficult to explain, but it probably results from blockage at a lower level, where œdema of the interstitial tissue is a marked feature. Lower in the nephron marked degeneration of the epithelium of the ascending and descending limbs of Henle is seen. Not only is this apparent, but there is also widespread cast formation. The casts appear to be largely albuminous and stain blue or red with Masson, although some variation is seen. Their probable composition is to form the subject of a special study. The collecting tubules also show epithelial degeneration and cast formation, and stripping of the epithelium from the basement membrane. At this level there is marked œdema of the interstitial tissue.

Renal histology, grade III. The changes here appear to be specially worthy of attention. As has been shown, the biochemical findings have features similar to those of traumatic uræmia. The histological appearances also have much in common. The glomeruli show little if any change. There is some albuminous fluid in the capsular space, but this is minimal.

The epithelium of the first convoluted tubules is not degenerate to any marked extent, although there is some loss of nuclear staining and in places nuclear pyknosis. Lying within the lumen of the tubules is an albuminous deposit. The second convoluted tubules show more degeneration, and in the majority cast formation is apparent. Some of the casts consist of epithelial debris; others appear to be albuminous. The general appearance of the cortex is shown in fig. 6. An albuminous deposit staining pink with eosin

PLATE XIV

EXPERIMENTAL OCCLUSION OF RENAL ARTERY

FIG. 3.—Rabbit no. 10, showing renal histology grade I. Marked degeneration of the convoluted tubules is seen, with comparatively normal glomeruli. H. and E. ×60.

FIG. 4.—Rabbit no. 17, showing renal histology grade II. A patchy distribution of degeneration of the convoluted tubules is seen. H. and E. ×60.
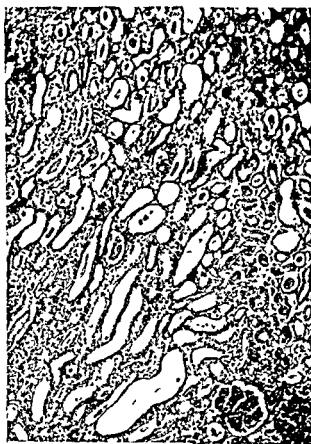
FIG. 5.—Rabbit no. 48, showing dilatation of the secreting tubules with occasional cast formation. H. and E. ×60.

FIG. 6.—Rabbit no. 33, showing renal histology grade III. The glomeruli and convoluted tubules show minimal degenerative changes. Casts are seen in the 2nd convoluted tubules. H. and E. ×60.

EXPERIMENTAL OCCLUSION OF RENAL ARTERY
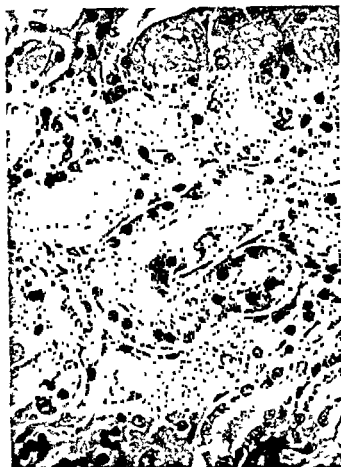
FIG. 7.—Rabbit no. 33, showing minimal degenerative change in the convoluted tubular epithelium, with albuminous (eosinophilic) fluid filling the lumen. This is comparable with what is seen in traumatic uræmia in man. H. and E. ×270.

FIG. 8.—Human case no. 2, showing the albuminous fluid lying in the lumen of the convoluted tubules. H. and E. ×145.

FIG. 9 —Rabbit no. 21, showing variability of cast formation comparable to what is seen in traumatic uræmia. H. and E. ×250.
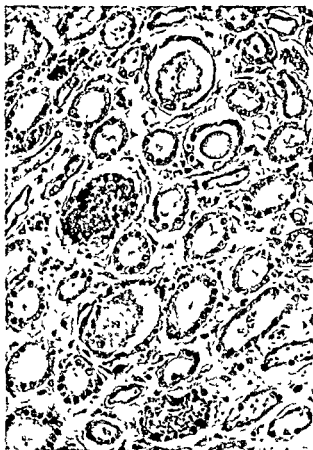
FIG. 10.—Human case no. 5, showing the variety of cast formation and the interstitial œdema which are features of human traumatic uræmia. Mallory's stain. ×145.

EXPERIMENTAL OCCLUSION OF RENAL ARTERY

FIG 11—Rabbit no 33, showing plication of epithelium in a collecting tubule (Compare with fig 12) H and E ×145

FIG 12—Human case no 3, showing plication of epithelium in a collecting tubule H and E ×145
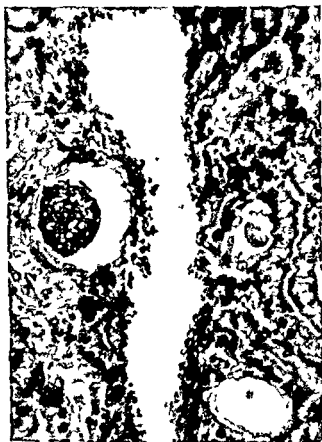
FIG 13—Rabbit no 6 showing a tubule about to rupture into a venous space This lesion is found with difficulty in the rabbit Mallory's stain ×250

FIG 14—Human case no 2 showing two tubules about to rupture into a venous space The lower has an ante mortem clot adhering to its projecting surface Mallory's stain ×65

PLATE XVII

EXPERIMENTAL OCCLUSION OF RENAL ARTERY
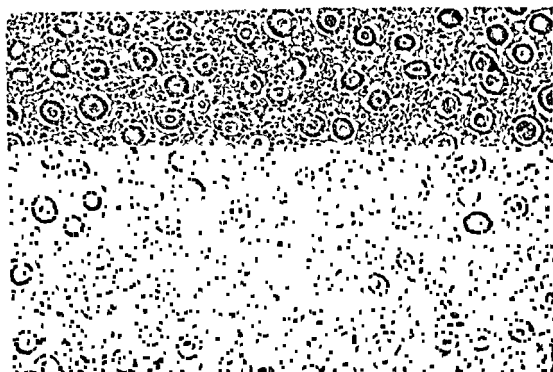
Fig 15 —Rabbit no 21, showing the pyramidal area in renal histology grade III
There is widespread degeneration of the loops of Henle and collecting tubules,
with interstitial œdema   Cast formation is of varied nature   H and E   ×95

Fig 16 —Rabbit no 24, showing the
early fibrosis associated with renal
histology grade IV occurring in the
pyramidal area   Van Gieson   ×115

Fig 17 —Rabbit no 31, showing a
localised area of the lymphocytic and
histiocytic infiltration seen in renal
histology grade IV   H and E   ×60

and loss of nuclear staining are marked features. The close similarity of this area in the rabbit to that in man may be seen by comparing figs. 7 and 8. Lower in the nephron further similarities occur. There is marked epithelial degeneration in the ascending and descending limbs of Henle in the pyramidal area (fig. 15) and again the type of cast present is strikingly similar (figs. 9 and 10). In both experimental and human cases the formation of the casts is of peculiar interest. Frequently they seem to be formed by albuminous deposition around detached epithelium. Around this in turn is seen either further detachment or the regeneration of new epithelium. This peculiar stripping of the epithelium, with a concertina effect known as plication, is common to both lesions (fig. 11). It has been described in man by Bywaters and Dible (fig. 12).

A further lesion which was found in all the human material examined was rupture of the tubules into the venous spaces, although this also occurred into the interstitial tissue. It was first described by Dunn, Gillespie and Niven (1941) and later by Bywaters and Dible. This lesion is found infrequently in the rabbit, but an example of a tubule about to rupture into a venous space is shown in fig. 13. This may be compared with what is seen in human cases (fig. 14).

Renal histology, grade IV. The histological material showing this lesion was obtained by killing the animal at the end of 28 days. That some degree of urea retention occurred in the early stages in this group of 12 animals has already been demonstrated (table V). The cortical areas show no change, but lower in the pyramidal area and boundary layer there is increased interstitial fibrosis with some residual œdema (fig. 16). A further change is the occurrence of groups of lymphocytes and histiocytes in areas which are apparently undergoing extensive repair (fig. 17).

Histology of animals killed earlier in the experimental period

A group of 4 animals which had had the routine clamping of one hour were killed when the blood urea commenced to fall. It was thought that by this means an earlier stage of the grade IV lesion might be observed. The results of daily blood-urea estimations are given in table VI.

The histological changes in these animals are perhaps not as marked as might have been expected in view of the urea retention. There is practically no change in the cortical area, except for some albuminous fluid and cast formation in the second convoluted tubules. In the area of the ascending and descending limbs of Henle the lesion is very patchy, and indefinite œdema of the interstitial tissue is seen. Degeneration of the tubular epithelium is again irregular, although cast formation is perhaps more widespread.

These findings suggest, first, that if the animal had been allowed to live the changes described under renal histology grade IV would have

been obtained and, second, that the most marked changes are in the lowest part of the nephron, *i.e.* the limbs of Henle.

TABLE VI

Serial blood-urea levels (mg. per 100 c.c.) after occlusion for 1 hour of the left renal artery and right nephrectomy

Rabbit no.	Initial weight (g.)	Blood-urea levels				
		Pre-operative	Days after clamping			
			1	2	3	4
82	2734	40	72	Killed	...	...
90	3178	33	90	136	95	Killed
91	2752	23	91	168	Killed	...
92	2336	30	108	264	114	Killed

DISCUSSION

The experiments described above establish three points; first, that irreversible damage to the kidney followed by death is caused in the unilateral nephrectomised rabbit if partial occlusion of the renal artery is maintained for 120 minutes; second, that the period of time for which the artery is occluded has a direct relationship to the mortality rate and period of survival; and third, that the first lesion produced is in the area last supplied with blood, namely the ascending and descending loops of Henle.

These results are perhaps slightly at variance with those of some previous authors, notably Scarff and Keele, who carried out experiments similar to ours except that the whole renal pedicle was clamped. Their animals were forcibly fed with water following operation, thus ensuring maximum diuresis. The histological picture which they describe conforms to what we have designated renal histology grade I or II. Again, their animals, although showing evidence of urea retention, were able to survive a two-hour compression for some days before dying or being killed. They do not appear to have found any variation in the histological picture in relation to the survival period.

On the other hand our findings largely support those of Van Slyke who, working with dogs, showed that the mortality and survival rates are largely dependent upon the length of time the clamp is applied to the renal artery. His evidence, when considered in relation to ours, indicates that the dog withstands renal ischæmia for longer periods than the rabbit. Evidence of the length of time necessary to cause irreversible damage in man is lacking, but it would appear to be more than six hours.

A histological picture somewhat similar to our renal histology grade II is described by McEnery *et al.*, who were also struck by the variety of the areas affected. Our grades I and II lesions are some-

what similar to those described by Ash (1933) and Dunn and Montgomery (1941) in discussing the cortical necrosis of the kidney occurring in toxæmia of pregnancy. Ash states that the essential feature appears to be thrombosis of the arteries, especially the intralobular vessels, accompanied by hyperæmia, exudation and œdema. Both he and Dunn and Montgomery attribute this appearance to ischæmia. Cases of cortical necrosis have been attributed to ischæmia in conditions other than toxæmia of pregnancy. Godwin and McCall (1941), for example, report its occurrence in a man who died with peritonitis following perforation of a gastric ulcer, and McFarlane (1941) found it after multiple fractures with internal hæmorrhage. Both are agreed that the renal condition was probably due to ischæmia.

We had some difficulty in deciding whether it was possible for blood to pass when the clamp was in position. We were able to examine sections of renal arteries in 4 animals (not included in the experimental series above) which had died from the anæsthetic during operation. From these we found that the occlusion of the artery was not entirely complete, and it was thought possible that a small quantity of blood could pass whilst the clamp was in position.

Although we found that the histological appearances vary with the survival period, the reason for the variability of the latter is not apparent. Three factors may, however, be operative. First, although every effort was made to produce a standard compression there may have been some variation in the amount of blood allowed to pass. Second, the clamping may have produced damage to the artery so that, after release, the blood flow varied from animal to animal. Third, spontaneous spasm of the artery may have occurred after the clamp had been released. During the course of the experiment we had visual evidence that, whilst the artery and vein were being dissected or when traction on or handling of the kidney was in process, the renal artery went into spasm. This was associated with a notable colour change. The normal plum colour of the kidney was turned to pale yellow, frequently of patchy distribution. At the same time, the kidney was reduced in size and the capsule became wrinkled. This appearance continued until the spasm of the artery disappeared, when the kidney increased in size and the colour gradually returned, although often this appeared to be rather more cyanotic than before. An exactly comparable phenomenon was observed after clamping the renal artery, and was so striking that it was subsequently taken as evidence of the success of the clamping manœuvre.

The ease with which spasm of the renal artery can occur is perhaps not as widely recognised as it should be. Marshall and Crane (1923), Stoll and Carlson (1923-24) and Starr (1926), in observing the kidney after operative procedures in experimental animals, were troubled by the ease with which this could occur. Schroeder and Steele (1940) also found that in anæsthetised dogs made super-sensitive to adrenaline,

the renal vessels responded to what would normally have been a sub-normal dose and passed into a long-lasting constriction. It is therefore possible that spontaneous spasm may play some part in producing anomalous results.

The fact that the first lesion to occur as the result of clamping is degeneration of the ascending and descending limbs of Henle requires more detailed consideration. Dunn and Polson studied the blood supply to the kidney in the rabbit, and demonstrated that the ascending and descending limbs of Henle are the last to be supplied with blood. It follows that a reduction in blood flow would lead to this area being supplied with blood poor in oxygen. Since the limbs of Henle require considerable energy for the maintenance of their function in activity, these structures would appear to be particularly vulnerable to anoxia.

Study of the animals which recovered, e.g. those showing renal histology grade IV, reveals almost complete absence of change in the cortical zone. The fact that healing changes are occurring in the boundary area and in the ascending and descending limbs of Henle is perhaps the strongest evidence that this is the first part of the kidney to be damaged by a failing blood supply. This is confirmed by animals killed either at the commencement of the fall of blood urea or immediately afterwards. It would appear, therefore, that these changes are probably caused by a partial reduction of blood flow rather than its complete cessation, since this latter leads to changes described as renal histology grade I.

We know that when a rabbit develops an almost complete renal ischæmia changes similar to those of renal histology grade I are found. For example in rabbit no. 66, through an oversight, the clamp was not removed and the blood urea, which on the day of operation was 42 mg. per 100 c.c., had risen on the following day to 156 mg. It died approximately thirty-six hours after operation.

Summary

1. Temporary partial occlusion of the remaining renal artery in the unilateral nephrectomised rabbit leads to renal changes comparable to those seen in human traumatic uræmia.

2. The length of time during which there was occlusion of the artery bears a direct relationship to the mortality and survival rates of these animals.

3. The first renal lesion to occur in these circumstances is degeneration of the limbs of Henle.

REFERENCES

ASH, J. E. . . . . 1933. *Amer. J. Med. Sci.*, clxxxv, 71.

BAKER, S. L., AND DODDS, E. C. . 1925. *Brit. J. Exp. Path.*, vi, 247.

BEALL, D., BYWATERS, E. G. L., BELSEY, R. H. R., AND MILES, J. A. R. 1941. *Brit. Med. J.*, i, 432.

BEATTIE, J. M., AND DICKSON, W. E. C. 1908. A text-book of general pathology, London.

BYWATERS, E. G. L., AND BEALL, D. 1941. *Brit. Med. J.*, i, 427.

BYWATERS, E. G. L., AND DIBLE, J. H. 1942. This *Journal*, liv, 111.

BYWATERS, E. G. L. . . . . 1945. *Brit. Med. Bull.*, iii, 107.

CORCORAN, A. C., AND PAGE, I. H. 1943. *J. Exp. Med.*, lxxviii, 205.

COURNAND, A., RILEY, R. L., BRADLEY, S. E., BREED, E. S., NOBLE, R. P., LAUSON, H. D., GREGERSEN, M. I., AND RICHARDS, D. W. 1943. *Surgery*, xiii, 964.

DARMADY, E. M. . . . . . 1946-47. *Brit. J. Surg.*, xxxiv, 262.

DARMADY, E. M., SIDDONS, A. H. M., CORSON, T. C., LANGTON, C. D., VITEK, Z., BADENOCH, A. W., AND SCOTT, J. C. 1944. *Lancet*, ii, 809.

DUNN, J. S., GILLESPIE, M., AND NIVEN, J. S. F. 1941. *Lancet*, ii, 549.

DUNN, J. S., AND MONTGOMERY, G. L. 1941. This *Journal*, lii, 1.

DUNN, J. S., AND POLSON, C. J. . 1926. This *Journal*, xxix, 337.

EISENDRATH, D. N., AND STRAUSS, D. C. 1911. *Trans. Amer. Urol. Assoc.*, (1910), iv, 171.

GODWIN, B., AND McCALL, A. J. . 1941. *Lancet*, ii, 512.

GOLDBLATT, H. . . . . . . 1937-38. *Ann. Int. Med.*, xi, 69.

GOLDBLATT, H., LYNCH, J., HANZAL, R. F., AND SUMMERVILLE, W. W. 1934. *J. Exp. Med.*, lix, 347.

GOLDBLATT, H., WEINSTEIN, H., AND KAHN, J. R. 1941. *Ibid.*, lxxiii, 439.

GUTHRIE, C. C. . . . . . . 1910. *Arch. Int. Med.*, v, 232.

IGNATOWSKY, A. . . . . . 1905. *C.R. Soc. de biol.*, lvii, 130.

LAUSON, H. D., BRADLEY, S. E., AND COURNAND, A. 1944. *J. Clin. Invest.*, xxiii, 381.

McENERY, E. T., MEYER, J., AND IVY, A. C. 1926-27. *J. Lab. and Clin. Med.*, xii, 349.

McFARLANE, D. . . . . . 1941. This *Journal*, lii, 17.

MAEGRAITH, B. G., AND FINDLAY, G. M. 1944. *Lancet*, ii, 403.

MAEGRAITH, B. G., AND MACLEAN, F. J. 1942. *Brit. J. Exp. Path.*, xxiii, 239.

MARSHALL, E. K., Jr., AND CRANE, M. M. 1923. *Amer. J. Physiol.*, lxiv, 387.

MEYLER, L. . . . . 1935-36. *Acta Med. Scand.*, lxxxvii, 313.

PICKERING, G. W., AND PRINZMETAL, M. 1937-38. *Clin. Sci.*, iii, 211.

PHILLIPS, R. A., DOLE, V. P., HAMILTON, P. B., EMERSON, K., Jr., ARCHIBALD, R. M., AND VAN SLYKE, D. D.   1945-46. *Amer. J. Physiol.*, cxlv, 314.

ROWNTREE, L. G., FITZ, R., AND GERAGHTY, J. T.   1913.   *Arch. Int. Med.*, xi, 121.

SCARFF, R. W., AND KEELE, C. A.   1943.   *Brit. J. Exp. Path.*, xxiv, 147.

SCHROEDER, H. A., AND STEELE, J. M.   1940.   *J. Exp. Med.*, lxxii, 707.

VAN SLYKE, D. D., PHILLIPS, R. A., HAMILTON, P. B., ARCHIBALD, R. M., DOLE, V. P., AND EMERSON, K., Jr.   1944.   *Trans. Assoc. Amer. Physicians*, lviii, 119.

DE SOUZA, D. H. . . . . .   1900-01. *J. Phys.*, xxvi, 139.

STARR, I., Jr. . . . . . .   1926.   *J. Exp. Med.*, xliii, 31.

STOLL, J. E., AND CARLSON, A. J.   1923-24. *Amer. J. Physiol.*, lxvii, 153.

TOMB, J. W. . . . . . .   1942.   *J. Trop. Med.*, xlv, 125.

VERNEY, E. B., AND VOGT, M. .   1943-44. *Quart. J. Exp. Physiol.*, xxxii, 35.

WILSON, C., AND BYROM, F. B. .   1939.   *Lancet*, i, 136.

YORKE, W., AND NAUSS, R. W. .   1911-12. *Ann. Trop. Med. and Parasitol.*, v, 287.

THE ACTIVATION OF HÆMAGGLUTININS BY HUMAN SERUM *

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IN a series of experiments involving the titration of iso-agglutinins it was found that certain agglutinins gave a higher titration value when serum was used as diluent instead of normal saline. No description of this phenomenon was found in the literature, although there might be a connection between it and that described by Waaler (1939), who reported the occurrence of a factor in human serum which increased non-specifically the degree of the specific agglutination of sheep blood corpuscles. The ability of serum to increase the titration value of an agglutinin was of interest and might have some practical application ; therefore it was decided to carry out further investigations. The experiments were planned to determine whether all sera possessed this property, and also whether all agglutinins showed the increase in titre. A preliminary communication on the activation of hæmagglutinins has already been given (Boorman, Dodd and Morgan, 1945).

TECHNIQUE

The technique employed for the titration of agglutinins was essentially as described by Taylor and Ikin (1939). Serial dilutions of the serum under investigation were made with a Pasteur pipette graduated to deliver a constant volume (about 0 04 c.c.). To each of the serial dilutions an equal volume of a 2 per cent. suspension of the appropriate red cells was added and the tubes shaken. The titrations were made in duplicate and allowed to stand two or three hours, after which the red-cell deposit was agitated by tapping each tube sharply. A reaction was not recorded as negative until the contents of the tube had been examined microscopically. The presence of evenly distributed clumps of three or more cells was accepted as evidence of agglutination. Because Rh agglutination can very easily be broken down, special care was taken when reading titrations of anti-Rh agglutinins. The tubes were not tapped sharply but some of the sediment was gently removed with a Pasteur pipette, placed on a microscope slide and gently spread with the stem of the pipette.

The serum used as diluent in the titrations should not contain agglutinins or agglutinin-inhibiting substances (*e.g.* agglutinogens) which would interfere with the specific reaction under investigation. For the titration of anti-Rh, anti M and anti-N agglutinins it was not necessary to consider the anti-A and

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anti-B agglutinins in the diluent sera because the standard cells were always of group O ; but anti-A agglutinins could not be titrated in sera from persons belonging to groups B or O, nor anti-B agglutinins in sera of groups A or O. Unfortunately a serum which lacks either of the two agglutinins anti-A or anti-B contains the corresponding agglutinogen, which would tend to neutralise the antibodies under test. For the titration of anti-A agglutinins, therefore, a serum from a group-A donor which had no detectable inhibiting effect on the action of an anti-A serum was chosen. Similarly for the titration of anti-B agglutinins the serum used was obtained from a group-B donor and showed no inhibiting effect on anti-B agglutinins. No such precautions were taken in the selection of sera for use in titrating anti-Rh, anti-M or anti-N, as the corresponding agglutinogens have never been demonstrated in human serum.

In each experiment the titre of the agglutinin when titrated in serum was compared with the titre obtained using 0·85 per cent. saline as diluent. These titrations were always carried out at the same time and with the same cell suspension. The amplification of the agglutinin titre was expressed as the number of times the titre of the agglutinin diluted in serum was stronger than the titre in saline. For example, if the titre in serum was 256 and in saline 32, the amplification was recorded as 8.

RESULTS

Preliminary observations

In the first instance an anti-Rh iso-agglutinin was titrated in saline and in serum and the degree of agglutination throughout the series of dilutions was compared. Not only was a much higher titration value obtained by using serum as diluent, but also the agglutination was usually firmer and more complete than for the corresponding saline dilution.

The saline titre was next compared with that obtained using undiluted serum and serum diluted 1 : 2, 1 : 3, 1 : 5, 1 : 10 and 1 : 20. The results of the titrations are given in table I and show that serum diluted 1 : 2, 1 : 3 and 1 : 5 gave as good results as undiluted serum. On the basis of these results it was decided to use serum diluted 1 : 3 for further experiments as it was less viscous than undiluted serum and more economical.

The amplification effect was not destroyed by heating the diluting serum at 56° C. for half-an-hour or by storing it frozen solid.

Comparative effects of various sera as diluent

In order to determine whether all sera had an equal capacity to increase agglutination, a single anti-Rh agglutinin was titrated in 67 different sera (each diluted 1 : 3 as above). There was a wide range of amplification, values from 0 to 64 being obtained. Twelve of these titrations are shown in table II. Only once was there no amplification of the titre and even here there was a slight increase in the tightness of agglutination. As a result of these experiments it was decided to select certain sera for use as standard diluting fluids, and three sera were chosen which gave high amplification but possessed

TABLE I

Titration of an anti-Rh iso-agglutinin using as diluent normal saline, serum and various dilutions of serum

Titre	Saline	Serum					
		1:1	1:2	1:3	1:5	1:10	1:20
1	c c	c c	c c	c c	c c	c c	c c
2	v v	c c	c c	c c	c c	c c	v v
4	+ +	v v	v c	v v	v v	v v	v v
8	(+)(+)	v v	v v	v v	v v	++ ++	++ ++
16	w w	+ + v	v v	v ++	++ ++	++ ++	++ ++
32	— —	++ ++	++ ++	++ +	+ +	+ (+)	w w
64	...	+ +	+ +	+ +	(+)(+)	(+)(+)	— —
128	...	(+)(+)	(+)(+)	(+)(+)	w w	w w	...
256	...	w w	w w	w w	w w	— —	...
512	...	— —	— —	— —	...	...	...
Amplification		16	16	16	16	8	2

Key to all tables.

Read macroscopically.

    c = complete agglutination; surrounding fluid clear.
    v = visual agglutination; surrounding fluid pink.
    Hv = visual agglutination with some hæmolysis.
    H = hæmolysis.

Read microscopically.

    + + = very large clumps.
    + = large clumps.
    (+) = smaller clumps with many free cells.
    gw = slightly smaller clumps with many free cells.
    w = clumps of 4 or 5 cells.
    vw = evenly distributed clumps of 2 or 3 cells.
    — = no agglutination.

TABLE II

Titre of same anti-Rh iso-agglutinin in various sera

Serum dilution	Anti-Rh titre in saline	Anti-Rh titre in sera nos.											
		1	2	10	11	15	16	17	24	25	26	27	31
1:1	v	v	v	v	v	v	c	c	c	c	c	c	v
1:2	+	v	v	v	v	v	c	v	v	c	c	v	v
1:4	w	v	+	v	v	v	v	v	v	c	v	v	+
1:8	—	+	w	v	+	++	v	v	(+)	v	v	v	(+)
1:16	...	(+)	(+)	v	+	+	(+)	(+)	v	v	v	v	(+)
1:32	...	(+)	—	(+)	—	w	w	w	—	+	(+)	(+)	w
1:64	...	w	...	w	...	—	w	vw	...	w	w	(+)	—
1:128	...	—	...	—	...	—	—	—	...	—	—	w	...
1:256	...	...	...	...	...	...	...	...	...	...	...	vw	...
1:512	...	...	...	...	...	...	...	...	...	...	...	—	...
Amplification		16	4	16	4	8	16	16	4	16	32	64	8

agglutination. There was no instance of any increase in titre or tightening of agglutination with the non-immune antibody. In several cases the titrations of the agglutinins in saline and serum were

TABLE V

Comparative titres of immune anti-A and non-immune anti-B titres in saline and in serum

Case	Group of mother	Group of Infant	Anti-A		Amplification	Anti-B		Amplification
			Saline	Serum		Saline	Serum	
1	O	A	256,000	500,000	2	128	128	0
2	B	A	128	1000	8	...	...	...
3	A	B	...	...	...	1000	2000	2
4	O	A	32,000	128,000	4	512	512	0
5	O	A	256,000	4,000,000	16	8000	2000	0 (−4)
6	O	A	32,000	256,000	8	128	128	0
7	O	A	2000	2000	0	32	32	0
8	O	A	32,000	256,000	8	256	256	0
9	O	A	512	8000	16	128	128	0
10	O	A	512	4000	8	128	128	0
11	O	A	128,000	4,000,000	32	2000	2000	0
12	O	A	64,000	128,000	2	512	128	0 (−4)
13	O	A	512	4000	8	128	128	0
14	Group B patient given A blood		16,000	32,000	2	...	...	...
15	Group O patient given A blood		8000	32,000	4	1000	512	0 (−2)
16	Group O patient given A substance		256,000	2,000,000	8	128	128	0
17	Group O patient given A substance		1,000,000	16,000,000	16	4000	128	0 (−32)
18	Group O patient given A substance		128,000	1,000,000	8	512	512	0

done both before stimulation by the corresponding agglutinogen and at intervals throughout the course of the immune response. The results of one series of titrations of this kind are given in table VI. Group-A substance prepared from human ovarian cyst fluid (Morgan and van Heyningen, 1944) according to the method described by King and Morgan (1944) was given to a group-O individual. The results of the titration (table VI) show that there was no appreciable difference between the titre of the anti-A agglutinin in the two diluents for three days after the injection. By the 8th day, however, a definite tightening of the agglutination was observed and a higher titre was recorded for the dilutions made with serum. The sample collected on the 11th day after injection and titrated in saline and serum showed a striking difference in the titres of the anti-A agglutinin and contrasted sharply with the results of a similar titration for the anti-B agglutinin made on the same series of samples. The titration results show that there is no difference in titre to be observed through-

out for the anti-B agglutinin titrations made in saline or serum and there was no tightening of the B agglutination at any time during the course of antibody production.

TABLE VI

Comparison of titres in saline and in serum of a group-O individual given A-group specific substance both before injection and at intervals during the immune response

Antibody	Serum dilution	Before injection		Immediately after		Days after injection							
						1 day		3 days		8 days		11 days	
		Sal.	Ser.	Sal.	Ser.	Sal.	Ser.	Sal.	Ser.	Sal.	Ser.	Sal.	Ser.
Anti-A	1:1	v	v	c	c	v	v	H	H	H	H	H	Hv
	1:2	v	v	c	c	v	c	v	v	H	Hv	Hv	v
	1:4	c	c	c	c	c	c	c	c	Hv	v	v	v
	1:8	c	c	c	c	c	c	c	c	c	v	v	c
	1:16	c	c	v	v	c	v	c	o	o	c	o	c
	1:32	c	c	v	++	c	v	c	o	c	c	o	c
	1:64	v	v	++	+	v	v	o	o	c	c	o	c
	1:128	++	++	(+)	w	+	+	v	v	c	c	v	c
	1:256	+	(+)	gw	vw	gw	(+)	v	v	c	c	v	c
	1:512	(+)	gw	vw	−	vw	gw	+	+	c	c	v	c
	1:1000	w	w	−	...	−	vw	(+)	(+)	v	c	v	c
	1:2000	−	−	...	...	...	...	w	w	v	c	++	c
	1:4000	...	...	...	...	...	...	−	−	++	c	++	c
	1:8000	...	...	...	...	...	...	...	...	++	v	+	c
	1:16000	...	...	...	...	...	...	...	...	++	v	++	c
	1:32000	...	...	...	...	...	...	...	...	w	(+)	(+)	v
	1:64000	...	...	...	...	...	...	...	...	−	(+)	gw	v
	1:128000	...	...	...	...	...	...	...	...	−	−	w	++
	1:256000	...	...	...	...	...	...	...	...	...	...	−	gw
	1:512000	...	...	...	...	...	...	...	...	...	...		w
	1:1000000	...	...	...	...	...	...	...	...	...	...		w
	1:2000000	...	...	...	...	...	...	...	...	...	...		−
Anti-B	1:1	c	c	c	c	c	c	c	c	v	v	v	v
	1:2	c	c	c	c	c	c	c	v	v	v	v	v
	1:4	c	c	c	c	c	c	c	v	v	o	o	v
	1:8	v	c	c	c	c	c	c	c	c	c	c	v
	1:16	v	v	v	v	v	v	c	c	c	o	c	v
	1:32	++	++	++	++	++	++	v	++	++	++	v	v
	1:64	(+)	gw	+	(+)	(+)	(+)	(+)	(+)	+	+	++	v
	1:128	w	−	gw	w	(+)	w	(+)	w	(+)	(+)	+	(+)
	1:256	−		w	−	w	vw	w	vw	gw	w	gw	w
	1:512	...	...	...	...	−	...	−	−	vw	vw	vw	vw
	1:1000	...	...	...	...	...	...	−	−	−		−	
Amplification Anti-A . . .		0		0		2		0		2		8	
Anti-B . . .		0		0		0		0		0		0	

5. *Anti-A₁ (α₁) agglutinins.* Four naturally-occurring and 3 immune anti-A_1 (α_1) agglutinins were titrated with saline and serum as diluents. Two of the naturally-occurring agglutinins were found in the sera of persons belonging to group A_2B and 2 were prepared from group-B sera by absorption with A_2 erythrocytes. One of the anti-A_1 (α_1) immune agglutinins was found in the serum of a woman

of group A_2 after the delivery of an infant of group A_1 and one in the serum of a man of group A_2 who had been repeatedly transfused with group-A_1 blood. The third anti-A_1 (α_1) was a rabbit serum, prepared and described by Morgan and Watkins (1945). None of the naturally-occurring anti-A_1 (α_1) agglutinins showed any difference in titre when titrated with the two diluents, but a definite increase in titre and in tightness of agglutination was observed with all the immune anti-A_1 sera (amplifications 16, 2 and 8 respectively).

6. *Anti-M and anti-N agglutinins.* Two anti-M and one anti-N sera prepared by injection into rabbits of human erythrocytes of group OM and ON respectively were also tested. An increase in titre (representing amplifications of 16, 32 and 8 respectively) was observed in each instance, together with a definite degree of tightening of agglutination.

7. *Anti-O agglutinins.* One anti-O agglutinin naturally occurring in an ox serum and one immune anti-O agglutinin prepared in a rabbit (Morgan and Waddell, 1945) were titrated in both saline and serum in the usual manner. The ox serum was titrated against erythrocytes of groups A_1 and O and showed no increase in titre in serum with either type of erythrocyte. The rabbit anti-O serum was titrated against A_1, A_2, B and O erythrocytes. With the A_1 and B erythrocytes the titres in saline and serum were identical, being 2 in each case, but with O and A_2 erythrocytes the titres in serum increased from 8 to 32 and from 4 to 16 respectively.

DISCUSSION

As mentioned above, Waaler reported that the specific agglutination of sheep blood corpuscles was increased by the addition of human serum. He added 0·01 c.c. of human serum to a series of dilutions of anti-sheep-corpuscle serum, with the result that the agglutinin titre was increased from 2000 to 64,000. He did not connect the phenomenon with the fact that the antibodies were immune and apparently tested only animal sera. However, it seems possible that the factor which causes the amplification of human immune hæmagglutinins described in this paper is identical with that described by him.

Most human sera seem to contain this factor, although in widely varying amounts, and probably it is present in all sera. Since heating at 56° C. for half-an-hour has no effect on the amplifying power of a serum, the factors concerned are neither complement as a whole nor one of the heat-labile fractions.

The increase in titre with serum as diluent is not caused merely by the viscosity of the serum but by a specific factor contained in it. This is proved by the fact that while immune agglutinins show the increase there is no difference in the titre of naturally-occurring antibodies whether saline or serum is the diluent.

It was thought at first that this property might be very useful in

increasing the agglutinating power of weak · anti-Rh agglutinins. This would be of great importance in the diagnosis of hæmolytic disease of the newborn, because very weak antibodies are liable to be overlooked. While the combination Rh-negative mother and Rh-positive infant is merely suggestive of this disease, that of Rh-negative mother, Rh-positive infant and an anti-Rh agglutinin in the maternal serum is almost conclusive. Unfortunately, while it is true that weak antibodies are increased in titre, no case has yet been tested in which the presence of an antibody was demonstrated in the serum titration although none could be detected in saline. This is disappointing but not surprising, because in the undiluted serum to be tested for Rh antibody there is likely to be enough of the amplifying factor without the addition of that contained in the serum diluent; it is only at higher dilutions that the serum being tested for antibodies is too dilute to contain enough of the agglutinin-amplifying factor. However, the failure of this technique to detect weak Rh antibodies is of less importance now that various new methods of demonstrating their presence have been devised (Coombs *et al.*, 1945; Diamond and Abelson, 1945; Diamond and Denton, 1945; Wiener, 1945).

The most interesting point emerging from the investigation is the differentiation between immune and naturally-occurring agglutinins. It would seem to indicate a definite qualitative difference between them. This is not only of theoretical interest, but also of practical importance, especially in cases suspected of having had an incompatible blood transfusion. In such cases the presence of an immune anti-A, anti-B, anti-A_1, anti-O or anti-Rh agglutinin active at 37° C., is a strong indication that a hæmolytic reaction may have taken place. All anti-Rh agglutinins (only one reported exception—Diamond, 1942) are immune, but the anti-A, anti-B, anti-A_1 and anti-O agglutinins may be naturally occurring. That they are indeed immune in a given case has not hitherto been easy to establish unless repeated samples over a period of several weeks could be tested. With this technique, however, a positive result on a single sample demonstrating that the antibody is immune supports the clinical diagnosis of an incompatible transfusion. While it is true that in 77 of the 81 immune sera tested by us there was an increase in titre, in 21 of these the amplification was only 2. Since this increase is of the same order as the experimental variation, an amplification of 2 would not be taken as a positive result on a single sample, especially in the hands of a worker inexperienced in this technique. These results, moreover, were obtained with a serum chosen for its power of increasing the titre of an immune agglutinin; with a random serum as diluent the number of inconclusive cases would naturally be much greater. However, with a good serum as diluent, it is usually possible to tell whether or not a given agglutinin is immune.

Further work is necessary on the nature of the factor causing the enhancement of immune agglutinins. It is possible ·that it may be

connected with the mechanism responsible for Diamond's slide te[s] for Rh sensitisation and Wiener's conglutination phenomenon.

It seems likely that a study of this intrinsic difference betwee[n] naturally-occurring and immune antibodies might throw some lig[h] on the actual mechanism of agglutination.

SUMMARY

A property of normal human serum causing the enhancement [o] specific agglutination by immune agglutinins is described.

Eighty-one immune sera, comprising 49 anti-Rh, 7 anti-Rh sub group, 17 anti-A, 1 anti-B, 3 anti-A_1 (α_1), 1 anti-O, 2 anti-M and anti-N, were titrated in serum and in saline. In every instance ther[e] was an increase in the degree of agglutination in the serum diluent an[d] in 77 out of the 81 there was also a definite increase in titre.

Fourteen naturally-occurring agglutinins, comprising 5 anti-[A] 4 anti-B, 4 anti-A_1 (α_1) and 1 anti-O, were also examined. Ther[e] was no instance of an increase in the degree of agglutination or i[n] the titre when serum was used as diluent in place of saline.

The titration of a single anti-Rh agglutinin in 67 different ser[a] showed that they varied markedly in their power to amplify immun[e] agglutinins.

Some of the implications of this difference between immune an[d] naturally-occurring antibodies are discussed.

REFERENCES

AUBERT, E. F., BOORMAN, K. E., AND DODD, B. E.   1942.   This *Journal*, liv, 89.

BOORMAN, K. E., DODD, B. E., AND MORGAN, W. T. J.   1945.   *Nature*, clvi, 663.

CAPPELL, D. F. . . . . .   1944.   *Glasgow Med. J.*, cxlii, 125.

COOMBS, R. R. A., MOURANT, A. E., AND RACE, R. R.   1945.   *Brit. J. Exp. Path.*, xxvi, 255.

DIAMOND, L. K. . . . .   1942.   *New Engl. J. Med.*, ccxxvii, 857.

DIAMOND, L. K., AND ABELSON, N. M.   1945.   *J. Lab. and Clin. Med.*, xxx, 204.

DIAMOND, L. K., AND DENTON, R. L.   1945.   *Ibid.*, xxx, 821.

KING, H. K., AND MORGAN, W. T. J.   1944.   *Biochem J.*, xxxviii, (Proc.), x.

MORGAN, W. T. J., AND VAN HEYNINGEN, R.   1944.   *Brit. J. Exp. Path.*, xxv, 5.

MORGAN, W. T. J., AND WADDELL, M. B. R.   1945.   *Ibid.*, xxvi, 387.

MORGAN, W. T. J., AND WATKINS, W. M.   1945.   *Ibid.*, xxvi, 247.

TAYLOR, G. L., AND IKIN, E. W. .   1939.   *Brit. Med. J.*, i, 1027.

WAALER, E. . . . . . .   1939.   Proc. of 3rd Internat. Cong. f[or] Microbiol, *New York*, p. 777.

WIENER, A. S. . . . . .   1945.   *J. Lab. and Clin. Med.*, xxx, 662.

ON CERTAIN PROPERTIES OF ANTISERA PRE-PARED AGAINST HUMAN SERUM AND ITS VARIOUS PROTEIN FRACTIONS: THEIR USE IN THE DETECTION OF SENSITISATION OF HUMAN RED CELLS WITH "INCOMPLETE" Rh ANTIBODY, AND ON THE NATURE OF THIS ANTIBODY

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A METHOD has previously been described for detecting non-agglutinating Rh sensitisation of human red blood cells by exposing the washed sensitised cells to an anti-human-globulin serum (Coombs, Mourant and Race, 1945a, 1945b and 1946). Non-agglutinating Rh sensitisation may be caused by an "incomplete" Rh antibody (Race, 1944). This type of antibody, provisionally called "incomplete", sensitises homologous cells but does not agglutinate them under the usual conditions employed for carrying out agglutination tests.

It had been presumed that the rabbit anti-human-globulin serum reacted specifically, in the immunological sense, with the globulin of the Rh-sensitising antibody once it had been adsorbed specifically on to the homologous red cells, and in this way brought about their agglutination.

In order to investigate the best way of producing antisera suitable for this test, the sera detailed in table I were examined for their ability to agglutinate such sensitised cells. Also with the aid of the sera detailed in table II further observations have been made on the nature of the "incomplete" Rh antibody.

Methods

The antisera prepared as detailed in tables I and II were inactivated at 56° C. for half-an-hour and thoroughly absorbed with A, B and O human red cells. They were then examined for their ability to agglutinate R_2r cells $\left(\text{corresponding to Fisher's antigenic or genetic formula } \dfrac{cDE}{cde}\right)$, which had previously been fully sensitised with the "incomplete" anti-D Rh antibody. The R_2r cells after sensitisation with the "incomplete" antibody were washed three times in saline to remove the human serum, and were then re-suspended in

saline to give a 2 per cent. suspension. In the titrations of the antisera, two drops of this 2 per cent. suspension of sensitised cells were added to two drops of increasing dilutions of antisera. The results were read microscopically after one hour at 37° C.

TABLE 1

Suitability of antisera for agglutination of sensitised cells

Rabbit antisera	Antigen	Course of immunisation	++ agglutination with sensitised cells
1	Human serum	1 course : 4 injections i/p 2 c.c. at 4-day interval : bled 14th day	1 : 16
2	„ „ group O	1 course : 4 injections i/v 1·5 c.c. at 3-day interval : bled 12th day	1 : 16
3	„ „ „ O	1 course : 4 injections i/v 1·5 c.c. at 3-day interval : bled 12th day	1 : 64
4	„ „ „ O	2 courses : 4 injections i/v 1·5 c.c. at 3-day interval : bled 10th day (Mixed sera from two rabbits)	1 : 256
5	„ „	1 course : 6 injections i/v 1/10 c.c. daily : bled 9th day (Absorbed at optimum proportions with sheep serum)	Over 1 : 64
6	Alum - precipitated human serum	1 injection i/m 5 c.c. into each buttock : bled 14th day	1 : 32
6b	„ „ „	2 injections i/m 5 c.c. into each buttock : bled 14th day	1 : 512
7	„ „ „	2 injections i/m 5 c.c. into each buttock : bled 14th day	1 : 512
8	„ „ „	2 injections i/m 5 c.c. into each buttock : bled 14th day	1 : 128
9	„ „ „	2 injections i/m 5 c.c. into each buttock : bled 14th day	1 : 512
10	„ „ „	2 injections i/m 5 c.c. into each buttock : bled 14th day	1 : 128
11	Human γ globulin	From Dr Record, Lister Institute 1 c.c. antiserum = 0·425 mg. globulin (constant antibody)	1 : 64
12	Human globulin (50 per cent. sat. ammon. sulph.)	2 courses : 5 injections i/p 1 c.c. at 3-day intervals : bled 9th day	Over 1 : 64
13	Human pseudo-globulin (33·50 per cent. sat. ammon. sulph.)	1 course : 6 injections i/v 5 mg. protein: bled 9th day	1 : 16
14	Pneumococcus type 2	Wellcome pneumococcus type 2 anti-serum *	None
15	Typhoid bacilli	Anti-H titre 1 : 15,000 *	None
16-20	...	Normal rabbit sera *	None

* Lowest dilution tested 1 : 4.

EXPERIMENTAL RESULTS

Experiment I

Table I shows the titres at which these various rabbit antisera gave a ++ microscopic agglutination with R_2r cells *fully sensitised* with "incomplete" anti-D Rh antibody. A detectable reaction

however was given at a much higher dilution in each case. The smallest amount of antiserum giving a $++$ reaction is considered as containing one agglutinating dose, and for routine testing eight agglutinating doses are used. Serum no. 4, for example, gives a $++$ reaction at 1 : 256 and for routine tests is used at a dilution of 1 : 32.

None of the sera in tables I and II, after absorption with A, B and O cells, had any visible action on unsensitised human cells.

Observations on table I

1. Antisera produced against whole human serum are satisfactory for this test. Two courses of four injections each may be needed to produce a sufficiently high-titred serum. It is desirable to use group O human serum as antigen in order to avoid the production of unwanted anti-A and anti-B, which may result from the presence of dissolved A and B antigens in the human serum.

2. Rabbit anti-alum-precipitated-human-serum sera, prepared according to the method of Proom (1943), prove to be very satisfactory. Two inoculations seem adequate to produce a serum of sufficiently good titre.

3. High-titred anti-typhoid and anti-pneumococcus type 2 rabbit sera are shown to have no visible effect on cells sensitised with the "incomplete" anti-D Rh antibody. This presumably shows that the reaction is not due to non-specific colloidal effects of immune sera in general, as has been suggested to us by some workers.

4. Two rabbit anti-alum-precipitated-horse-serum sera, not given in the table, were shown to agglutinate cells sensitised with the "incomplete" anti-D Rh antibody, but only at a very low titre. This is probably analogous to the cross reactions observed with the precipitin test between human and horse serum.

Further observations

On treating a human serum containing the "incomplete" Rh antibody with ammonium sulphate, it was found that the fraction precipitated by 33 per cent. saturation, *i.e.* the fraction containing most of the γ globulin, contained most of the "incomplete" Rh antibody. The fraction of the anti-Rh serum precipitated between 33-50 per cent. saturation with ammonium sulphate, which contains most of the α and β globulins as well as some of the γ globulin, also sensitised the R_2r cells, although not as strongly as did that fraction precipitated below 33 per cent. saturation. The albumin fraction of the same anti-Rh serum did not sensitise the cells at all. From this it appeared likely that the "incomplete" anti-D Rh antibody was contained mainly if not entirely in the γ globulin fraction of human serum.

Surprisingly it was found that an antiserum produced in a rabbit against a (Cohn) albumin fraction of human serum strongly

agglutinated R_2r cells sensitised with the "incomplete" antibody. If the antiserum to the (Cohn) albumin were specific for the albumin fraction of human serum, it would not be expected to react, especially at a high titre, with the γ globulin of the "incomplete" Rh antibody.

TABLE

Neutralisation of antisera with different

Antisera	Antigens injected into rabbits to produce the antisera	++ agglutination reaction with cells sensitised with "incomplete" antibody	Equivalents of antisera found by precipitin flocculation tests by method of optimal proportions
21	Human albumin fraction	1 : 256	1 cc. antiserum ≡ 7·8　mg. human albumin ≡ 0·96 mg. human α and β globulin ≡ 0·24 mg. human γ globulin
22	Human albumin fraction	1 : 16	Antiserum too weak to demonstrate definite reacting zones
23	Human α and β globulin	1 : 256	1 c.c. antiserum ≡0·015 mg. human albumin ≡3·9　mg. human α and β globulin ≡3·9　mg. human γ globulin
24	Human α and β globulin	1 : 128	Antiserum too weak to demonstrate definite reacting zones
25	Human γ globulin	Weak reaction at 1 : 4	,,　　,,　　,,　　,,　　,,
26	Human γ globulin	Weak reaction at 1 : 4	,,　　,,　　,,　　,,　　,,
27	Human γ globulin	1 : 64	1 c.c. antiserum ≡1·95　mg. human α and β globulin ≡0·425 mg. human γ globulin
4	Human serum group O (as no. 4 in table I)	1 : 256	1 c.c. antiserum ≡0·48 mg. human albumin ≡7·8　mg. human α and β globulin ≡7·8　mg. human γ globulin
6b	Alum-precipitated human serum (as no. 6b in table I)	1 : 512	1 c.c. antiserum ≡0·03 mg. human albumin ≡0·24 mg. human α and β globulin ≡0·06 mg. human γ globulin

This anti-albumin serum also agglutinated cells sensitised with the fraction of anti-Rh serum precipitated below 33 per cent. saturation with ammonium sulphate. Here apparently the anti-albumin serum was reacting immunologically with the γ globulin of the Rh antibody. To explain this it was assumed that the anti-albumin serum was not specific for human albumin in that it reacted also with human γ globulin.

Experiment II

In order to investigate this question further the experiment summarised in table II was carried out. It was hoped to show that

II

protein fractions of human serum

Absorption or neutralisation of antisera	+ + agglutination reaction with sensitised cells after absorption of antisera
0·1 c.c. 1 : 4 antiserum+0·625 mg. human albumin	1 : 256
0·1 c.c. 1 : 4 antiserum+0·312 mg. human α and β globulin	1 : 32
0·1 c.c. 1 : 4 antiserum+0·078 mg. human γ globulin	...
0·1 c.c. 1 : 4 antiserum+0·312 mg. human albumin	1 : 16
0·1 c.c. 1 : 4 antiserum+0·312 mg. human α and β globulin	Weak reaction (at 1 : 8 and 1 : 16)
0·1 c.c. 1 : 4 antiserum+0·312 mg. human γ globulin	...
0·1 c.c. 1 : 4 antiserum+0·312 mg. human albumin	1 : 256
0·1 c.c. 1 : 4 antiserum+0·312 mg. human α and β globulin	1 : 128
0·1 c.c. 1 : 4 antiserum+0·312 mg. human γ globulin	...
0·1 c.c. 1 : 4 antiserum+0·312 mg. human albumin	1 : 128
0·1 c.c. 1 : 4 antiserum+0·312 mg. human α and β globulin	1 : 64
0·1 c.c. 1 : 4 antiserum+0·312 mg. human γ globulin	...
Antiserum too weak for absorptions	...
,, ,, ,, ,,	...
0·1 c.c. 1 : 4 antiserum+0·312 mg. human albumin	1 : 64
0·1 c.c. 1 : 4 antiserum+0·312 mg. human α and β globulin	1 : 16
0·1 c.c. 1 : 4 antiserum+0·312 mg. human γ globulin	...
0·1 c.c. 1 : 8 antiserum+0·312 mg. human albumin	1 : 256
0·1 c.c. 1 : 8 antiserum+0·312 mg. human α and β globulin	1 : 64
0·1 c.c. 1 : 8 antiserum+0·312 mg. human γ globulin	...
0·1 c.c. 1 : 4 antiserum+0·156 mg. human albumin	1 : 512
0·1 c.c. 1 : 4 antiserum+0·156 mg. human α and β globulin	1 : 128
0·1 c.c. 1 : 4 antiserum+0·156 mg. human γ globulin	...

the anti-albumin serum was non-specific in that it would react with both albumin and γ globulin, and that the basis of this test for demonstrating the " incomplete " antibody was a direct antibody-antigen reaction between the rabbit anti-human-globulin serum and the globulin of the Rh antibody adsorbed on the red cell. It was also hoped to show more definitely what fraction of human serum contained the " incomplete " Rh antibody.

Methods

In this experiment the rabbit antisera enumerated in table II were used. Antisera 21-26 were prepared by giving the rabbits one course of five injections intraperitoneally of 0·1 mg. protein at four-day intervals. The rabbits were bled on the tenth day after the last injection. The human-serum-protein fractions used for the immunisation as well as for the neutralisation procedure and as antigens in the precipitin tests were (Cohn) fractions prepared at Harvard University. Antiserum no. 27 was prepared by Dr B. R. Record and Dr Margaret E. Mackay of the Lister Institute, who used as antigen human γ globulin prepared according to the method of Kekwick (1940).

The antisera, after being heat-inactivated at 56° C. for half-an-hour and absorbed with A, B and O cells as usual, were tested by the precipitin floccula-tion test. The sera diluted 1 : 5 were tested against human albumin, α and β globulin and γ globulin fractions to see if they were specific for their respective antigen protein fractions. The equivalents of the antisera to the respective protein fractions were found by the method of optimal proportions using constant antibody (Dean and Webb, 1926).

The antisera were then tested, before and after neutralisation with the different protein fractions of human serum, for their ability to agglutinate R_2r cells sensitised with the " incomplete " Rh antibody. The neutralisation procedure consisted simply of adding the protein solution to the antiserum and incubating for half-an-hour at 37° C. If a precipitate formed it was removed by centrifugation before the serum was further used. The concentra-tions of the different protein fractions used in the neutralisations had no visible effect on the unagglutinated sensitised cells. A higher concentration of albumin would presumably have caused unagglutinated sensitised cells to agglutinate, as occurs in Diamond's test for the " incomplete " antibody (Cameron and Diamond, 1945).

Observations on table II

1. Antisera prepared against the different (Cohn) human-serum-protein fractions were shown not to be specific for their respective fractions, as judged by the precipitin flocculation test.

2. Neutralisation of the different sera with albumin in no way reduced the visible agglutinating titre of any of these sera against cells sensitised with " incomplete " anti-D Rh antibody.

3. Neutralisation of the different sera with γ globulin completely removed the ability of all these sera to agglutinate cells sensitised with the " incomplete " anti-D Rh antibody.

4. Neutralisation of the different sera with the α and β globulin fractions, well in the zone of antigen excess, only slightly reduced the agglutinating power of these sera for the sensitised cells. This slight reduction in the agglutinating power may be due to traces of γ globulin in the α and β globulin fraction used for neutralisation, or, on the other hand, a small amount of the anti-Rh antibody may be contained in the α and β globulin fraction of human serum.

5. From the above it would seem evident that the antibody in the rabbit serum which agglutinates cells sensitised with the " in-complete " Rh antibody is an anti-γ globulin antibody, and that the " incomplete " anti-D Rh antibody is present in the γ globulin

fraction of human serum. However it cannot be stated that a small amount of the "incomplete" anti-D Rh antibody is not contained in the α and β globulin fraction (see Cohn *et al.*, 1944).

6. The isolated (Cohn) γ globulin fraction of human serum used in this experiment seemed a very poor antigen *in vivo*. An antibody capable of reacting with γ globulin was produced in rabbits immunised with (Cohn) albumin and α and β globulin fractions; yet this same antibody was not neutralised *in vitro* by (Cohn) albumin or α and β globulin fractions, but only by (Cohn) γ globulin.

7. It will be noticed that the anti-γ globulin antibody equivalents of the sera did not correspond with the titres of the sera for agglutinating sensitised cells. An explanation of this observation has not been attempted.

SUMMARY

1. Methods are described for producing rabbit anti-human-globulin sera for the detection of sensitisation of human red cells with "incomplete" Rh antibodies.

2. The serological evidence put forward strongly suggests that the "incomplete" anti-D Rh antibody is present in the γ globulin fraction of human serum; a small amount may however be present in the α and β globulin fraction. The agent in rabbit antisera causing agglutination of cells sensitised with the "incomplete" anti-D Rh antibody appears to be an anti-globulin antibody, mostly if not entirely anti-γ globulin, in spite of the fact that it may be elicited by highly purified preparations of other serum proteins.

REFERENCES

CAMERON, J. W., AND DIAMOND, L. K.   1945. *J. Clin. Invest.*, xxiv, 793.

COHN, E. J., ONCLEY, J. L., STRONG, L. E., HUGHES, W. L., Jr., AND ARMSTRONG, S. H., Jr.   1944. *Ibid.*, xxiii, 417.

COOMBS, R. R. A., MOURANT, A. E., AND RACE, R. R.   1945a. *Lancet*, ii, 15.

   ,,   ,,   ,,   ,,   1945b. *Brit. J. Exp. Path.*, xxvi, 255.

   ,,   ,,   ,,   ,,   1946. *Lancet*, i, 264.

DEAN, H. R., AND WEBB, R. A. .   1926. This *Journal*, xxix, 473.

KEKWICK, R. A. . . . . .   1940. *Biochem. J.*, xxxiv, 1248.

PROOM, H. . . . .   1943. This *Journal*, lv, 419.

RACE, R. R. . . . . . .   1944. *Nature*, cliii, 771.

PENICILLIN AND *B. ANTHRACIS*

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THIS paper describes experiments, carried out both *in vitro* and *in vivo*, on the action of penicillin against *Bacillus anthracis*.

LITERATURE

In their report on penicillin Abraham *et al.* (1941) list *B. anthracis* among the organisms showing the same degree of sensitivity towards penicillin as the staphylococcus. Since then there have been a number of papers and reports on the use of penicillin in the treatment of experimental anthrax infections in animals and accidental infections in man. Heilman and Herrell (1944) infected mice with a suspension of anthrax washed off a 24-hour agar slope which was presumably a mixture of sporing and vegetative forms. The mice were given about 10,000 M.L.D. ($2 \cdot 8 \times 10^5$ organisms) subcutaneously and treatment with penicillin was started 16 hours later. This consisted of a total daily dose of 1000 units given in 5 doses during the 24 hours and continued for 12 days. Eleven out of 20 mice survived and all controls died. They noted that all mice in the treated group that developed any local lesion eventually died. Mice given only 10 M.L.D. and the same treatment started 1 hour after infection all survived. Sterne (1945) infected guinea-pigs with 100 M.L.D. of anthrax spores by subcutaneous injection and started treatment 24 hours later, by which time a local lesion measuring 7×5 cm. had developed. The penicillin was given in 4-hourly injections subcutaneously. With a total daily dose of 200 units given for 7 days, one of ten animals survived. Where the daily dose was 800 units all survived. All the controls died. Julianelle (1944, personal communication) successfully treated very small groups of mice infected with broth cultures of anthrax by means of a few large doses of penicillin. He found that a single dose of 400 units was effective if given up to 5 hours after infection but not if given later than this. American workers (personal communication from Camp Detrick, Frederick, Md., U.S.A.) have obtained successful results in the treatment of cutaneous infection in monkeys and subcutaneous infections in rabbits and mice. Monkeys were given 750 units 3 hourly for 72 hours after their blood cultures had become positive following the application of anthrax spores to their abraded skin. Rabbits given up to 100,000 spores subcutaneously survived if given 1000 units 3 hourly for 3 days, starting 1 hour after infection. A much larger series of mice was used and in the majority a subcutaneous injection of 5000 spores (100-1000 M.L.D.) was the infecting dose. Treatment consisted of 3-hourly injections starting 24 hours after infection and continuing for 7 days. With single doses ranging from 1250 down to 78 units the results were similar. About 50 per cent. of the animals survived. It was noted, however, that in some treated mice a local lesion developed and subsequently regressed, with recovery of the animal. All the untreated control mice died. Mitchell and Chapman (1944, personal

communication) infected small groups of mice with 200-400 spores and gave one of two different doses of penicillin 8 times daily for 5 or 10 days—a dose of 7·5 units saved all the mice, whereas 3·5 units saved only 2 out of 5. Treatment was started 3 hours after infection but if delayed for 24 hours the dose of 7·5 units saved only 3 out of 5 mice. In general these results indicate that, despite the apparent sensitivity of *B. anthracis* to penicillin, experimental infections do not yield as readily to treatment as streptococcal infections.

With regard to human infections Murphy *et al.* (1944) report success in the treatment of malignant pustules in three patients. They recommend a daily dose of 200,000-400,000 units given over three days. In their own cases there was a striking response to such treatment and the local signs in the lesions are recorded as having shown signs of abatement within 12 hours of beginning treatment. A much larger series of cases has been reported by Ellingson *et al.* (1946, personal communication). Treatment was successful in all cases with a total dosage of 1-1½ million units in 3-hourly doses over 5-7 days. Local packs of penicillin were also given. The lesions dried up about the 4th day and cultures from them became sterile much earlier.

EXPERIMENTAL

Studies in vitro

Inoculum size. Early reports of work on staphylococci and streptococci indicated that the sensitivity of these organisms to solutions of penicillin in ordinary culture media was to a large extent independent of the size of the original inoculum. Anomalous results in the early experiments with *B. anthracis* were soon correlated with irregularities in the size of inocula used.

A series of experiments was therefore performed in which the size of the bacterial inoculum was titrated against different amounts of penicillin in the culture medium. Cultures were made in 1 per cent. Lemco broth containing known concentrations of penicillin. The penicillin was a commercial preparation and the strength of the solutions was estimated against a solution of a standard barium salt kindly supplied by Dr N. G. Heatley.

The bacterial inocula consisted of washed spores. Part of a stock spore suspension (3×10^{10} per ml.) prepared from an agar culture was fully lysed to remove all remnants of vegetative forms, washed three times in distilled water and stored at a concentration of about 2×10^{10} per ml. Samples of this suspension, which consisted for the most part of single free spores, were diluted appropriately for the experiments and counted by the method of Miles and Misra (1938), which gave results accurate to within 10 per cent. The strain used was a standard laboratory strain obtained from Dr R. L. Vollum—the "Monroe" strain of the School of Pathology, Oxford. Originally obtained from the Veterinary College, it had been subcultured weekly for 12 months before it was received here in 1941, and had since had many artificial subcultures and occasional passages through mice and guinea-pigs. The spores were added to the broth containing the penicillin to make a final known concentration of both organisms and penicillin and incubated for 24 hours at 37° C. At the end of the

period the presence or absence of growth was recorded by naked-eye observation.

A summary of the results is presented in table I and those of a wider range of experiments are recorded graphically in the accompanying figure. The presence or absence of growth after 24 hours is clearly a function of the concentration of the penicillin and the number of spores originally present.

TABLE I

Relationship between inoculum size and concentration of penicillin in determining growth of B. anthracis *after 24 hours at 37° C.*

Concentration of spores per ml. of broth	Concentration of penicillin (units/ml. of broth)					
	10	5	2	1	0·5	0
1×10^8	+	+	+	+	+	+
1×10^7	+	+	+	+	+	+
5×10^6	−	−	+	+	+	+
1×10^6	−	−	−	+	+	+
5×10^5	−	−	−	−	+	+
1×10^5	−	−	−	−	−	+

+ = growth
− = no growth

Sensitivity of other strains of B. anthracis. A number of strains of *B. anthracis* were tested for their sensitivity to penicillin *in vitro*. With inocula of equal size it was impossible to detect any differences in the susceptibility of the following strains : (1) standard laboratory strain after many in-vitro cultures ; (2) standard laboratory strain after one monkey passage ; (3) standard laboratory strain after three monkey passages ; (4) strain 99 (obtained from U.S.A.) ; (5) strain 99 isolated from accidental infection (obtained from U.S.A.) ; (6) recently isolated cattle strain ; and (7) avirulent strain for vaccine production (Weybridge).

Action of penicillin on anthrax spores. A suspension of 1×10^6 spores per ml. was incubated in broth with 2 units of penicillin per ml. Immediately after mixing, a sample suitably diluted for counting gave the expected number of colonies. After 18 hours at 37° C. no colonies grew from the sample diluted for counting. Incubation for the same period in saline containing penicillin brought about no change in the total spore count.

Suspensions of spores of 1·2×10⁵/ml. were made up in broth with and without the addition of penicillin (2 units/ml.). Samples were removed and diluted 100 times for counting after incubation at 37° and 4° C. At 37° C. the counts remained unaltered for 2 hours. In the tube containing the penicillin there was a slight fall at the end of 3 hours and a sharp fall after 4 hours. Without penicillin the count remained the same for three hours and at the fourth hour

began to rise. Tubes kept at 4° C. showed no change, whether or not penicillin was present. It would appear that penicillin kills the organism after germination has been completed and the first divisions

FIGURE

THE RELATION BETWEEN INOCULUM-SIZE AND CONCENTRATION OF PENICILLIN CONTROLLING THE GROWTH OF ANTHRAX SPORES IN BROTH

+ = VISIBLE GROWTH 24 HRS. AT 37°C.
− = NO VISIBLE GROWTH 24 HRS AT 37°C.

CONCENTRATION OF SPORES PER ML BROTH

UNITS OF PENICILLIN PER ML BROTH
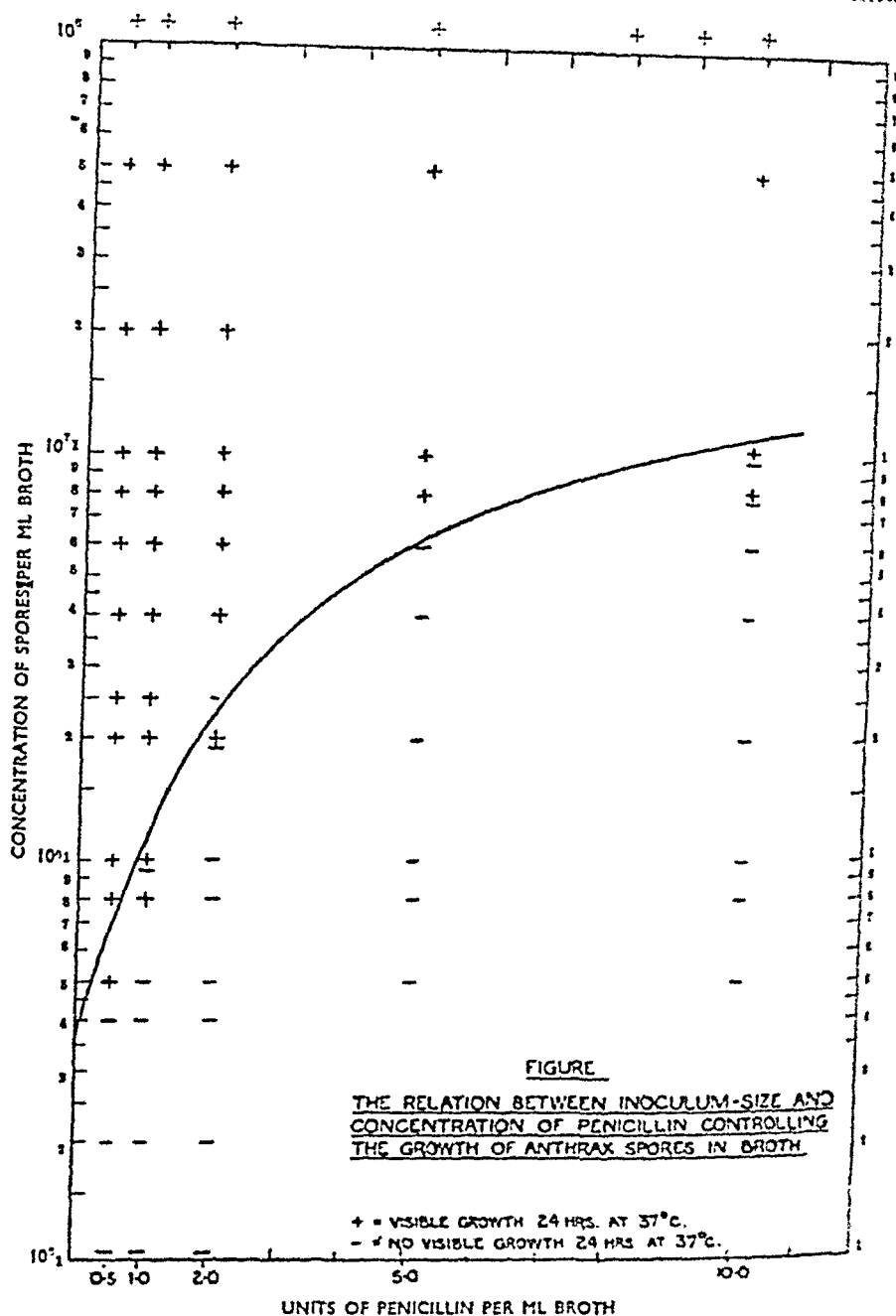
of the vegetative forms are taking place. Gardner (1945) has shown by direct microscopical observation that strong concentrations of penicillin will kill anthrax spores before there is any visible evidence

of germination. In minimal inhibitory doses the early vegetative forms emerging from the spores swell and die before their evolution is completed.

The first stage in the germination of anthrax spores takes place almost immediately on adding them to a rich medium such as tryptic meat broth. The spores are readily stained by Gram's method and are killed at a temperature of 65° C. Penicillin does not interfere with this process.

Penicillinase production. When a sufficiently large inoculum of spores is used to ensure growth in the presence of a given concentration of penicillin, examination of the supernatant culture medium after incubation shows that the penicillin has disappeared. Similar concentrations of penicillin with small inocula or broth alone were not affected by incubation at 37° C. for 24 hours.

Examination of the supernatant fluids of anthrax broth cultures showed that they were capable of destroying penicillin in solution. The active principle in this supernatant was found to be heat labile (destroyed rapidly at 65° C.), non-dialysable through cellophane and less active at low temperatures. It actually destroyed the penicillin and is almost certainly the enzyme penicillinase. Thus we have an organism which is both highly sensitive to penicillin in suitable concentrations and at the same time a natural producer of penicillinase. *Bacillus subtilis* behaves in the same way. It has been used to assay penicillin instead of staphylococci (Foster and Woodruff, 1944) and other strains have been used to produce good samples of penicillinase (Duthie, 1945).

Studies in vivo

An attempt was made to treat artificial anthrax infections of mice and rabbits with penicillin. Such infections once established in these two species invariably terminate fatally and the animals appear to offer little natural resistance to the disease. On the other hand, there may be no clearly recognisable foci of local infection and the animals appear to be overwhelmed by a gross bacteriæmia, so that it might be supposed that the infection would respond readily to an effective chemotherapeutic agent.

Rabbits. The animals were infected by the intravenous injection of anthrax spores suspended in saline. A dose of 100,000 spores invariably killed the 2-3 kilo rabbits that were used ; 50,000 spores killed approximately 75 per cent. of the animals. Fatal infections invariably caused death within 6 days. Animals which survived the smaller doses never showed any evidence of disease. Though outward manifestations of the disease might become evident only a few hours before death, blood cultures might be positive for as long as 60 hours before the animals died. At autopsy the animals showed little except the characteristic spleen and the hæmorrhagic changes in the lymph glands, varying in their intensity and distribution.

Sometimes there were appreciable pleural, pericardial or peritoneal exudates.

The animals were treated by intravenous injection of penicillin solution in saline—600 units every four hours, which is the weight-for-weight equivalent of a dose of 20,000 units for a 70-kilo man. Even this was probably inadequate, because penicillin disappears very rapidly from the blood stream of rabbits. After injection of 500 units no penicillin was detectable in the blood after 1 hour. After 1000 units there was penicillin in the blood stream after 1 hour but not after 2 hours. It made no difference if the penicillin was given intravenously, intramuscularly or subcutaneously.

Experiment 1. Five rabbits which had been given an intravenous injection of 50,000 spores were started on treatment (600 units four hourly) as soon as a smear from the blood became positive. As in untreated controls, death ensued within the next 48 hours and the course of the disease was unaffected.

Experiment 2. Twenty rabbits were infected with 100,000 spores ; 48 hours later, 10 of the animals were started on 4-hourly treatment with 600 units of penicillin. The 10 control animals were given saline so that all were submitted to the same amount of handling. The average survival times for the two groups were : (a) treated animals, 89·6 (140-70) hours ; (b) untreated controls 83·6 (140-57) hours. Again, treatment started late was without effect on the course of the infection.

Experiment 3. Twenty rabbits were infected with 100,000 spores. Sixteen rabbits received 4-hourly treatment with 600 units of penicillin, commencing four hours after the injection of the spores In one group of 8 the treatment was continued for three days and in the remaining 8 for five days. All four control animals were dead within 80 hours. In each group of the treated animals there were 4 survivors. Of those in the treated groups which died, only two did so in less than 80 hours and some lived as long as 8 days.

Started early, therefore, penicillin saved life after the injection of a fatal dose of anthrax spores. Its effect was not so striking as might have been expected.

Mice. A much larger series of experiments was performed with mice. Albino mice (18-20 g.) were used and housed singly or in pairs in small metal boxes. Penicillin was administered by subcutaneous injection under the skin of the back. The requisite number of units of penicillin was given in 0·1 ml. of saline. Most experiments were done with mice infected with anthrax spores. A dose of 3000 spores in 0·1 ml. of distilled water was given intraperitoneally to all mice. The suspension used in each experiment was counted and the calculated number of spores administered to any batch of mice ranged from 2800 to 3200. Deaths were recorded once daily. In the first series of experiments the mice were given 4-hourly injections of 10 units of penicillin starting four hours after infection and

continuing for 6 days. A dose of 10 units for a mouse is the weight-for-weight equivalent of a single dose of 35,000 units for a 70-kilo man. The results of four such experiments are summarised in table II.

TABLE II

Anthrax-spore infection in mice : results of treatment with 10 units of penicillin commencing four hours after infection and repeated four-hourly for six days

No of mice infected					Survivors on 9th day after infection	
Treated 110	.	.	.	.	35	31·8 per cent.
Untreated 110	.	.	.	.	20	18 3 ,,

The difference between the final mortality figures is not significant. On the other hand the mice in the two groups behaved differently. Most of the untreated mice were dead within 5 days and presented no outwardly visible sign of the disease. Those receiving treatment began to develop a striking œdema of the abdomen about the 4th or 5th day which progressed rapidly so that the animals could hardly walk ; they were obviously very ill. The death rate rose as soon as penicillin was stopped. By comparing the 50 per cent. mortality time from a curve of the death rate against time it can be seen that the penicillin significantly delayed the time of death in these animals (table III).

TABLE III

Anthrax-spore infection in mice : fifty per cent.-mortality time of control mice and mice treated with penicillin for 6 days

	50 per cent -mortality time
Treated mice	.	.	8 2 (6-11·2) days
Untreated mice	.	.	3 2 (2 6 3·9) days

Concurrent trials were made with penicillin administered in different ways These indicated that a short course of more intensive treatment in the early stages of the infection might be more effective (table IV). Each treated group received 300 units of penicillin, the equivalent of just over 1,000,000 units for a 70-kilo man.

Two further experiments were carried out with much larger doses of penicillin given during the first few hours of the infection. The results are given in table V.

Since this early intensive treatment seemed to be more effective another experiment was done to determine the amount of penicillin necessary to influence the mortality (table VI).

Three further experiments were then done to determine the importance of the time interval between infecting the animals and the

TABLE IV

Anthrax-spore infection in mice : effect of giving the same amount of penicillin over different periods of time

Treatment	Survivors on 9th day	
	Expt. 1	Expt. 2
50 units 4-hourly 1st day . .	16/20	13/30
25 units 4-hourly 1st and 2nd day .	12/20	...
10 units 4-hourly 1st-5th day . .	8/20	...
Untreated controls . . .	7/20	3/30

TABLE V

Anthrax-spore infection in mice : effect of two large doses of penicillin during the first 8 hours of infection

Treatment	Survivors on 9th day	
	Expt. 1	Expt. 2
500 units at 4th and 8th hour after infection	27/40	17/40
Untreated controls . . .	11/40	4/40

TABLE VI

Anthrax-spore infection in mice : effect of different doses of penicillin during the first few hours of infection

Treatment	Survivors on 9th day
500 units at 4th and 8th hour after infection .	17/40
100 ,, ,, ,, ,, ,, .	17/40
50 ,, ,, ,, . ,, ,, .	16/40
Untreated controls	4/40

initiation of penicillin treatment. All animals were given 300 units of penicillin in three doses of 100 units at two-hourly intervals. In this way a high concentration of the drug should be maintained within the mouse for 6 hours. The results are presented in table VII. Unfortunately, considerable variation in the death rate among the controls makes it impossible to compare the result of one experiment with that of another. It is possible to say, however, that treatment started up to 12 hours after infection significantly lowered the death

rate. Treatment started 20-24 hours after infection was without effect (table VII).*

<p style="text-align:center">TABLE VII</p>

Anthrax spore infection in mice: effect of treatment with penicillin started at different times after infection

Mean dose of spores   2800
Dose of penicillin: 300 units in three doses of 100 units (equivalent human dose · 1,050,000 units)

Expt	Interval (hours) between infection and start of treatment	Survivors on 9th day		Test of Independence
		Treated	Controls	
1	2 4 6 8	19/40 21/40 21/40 18/40		
	Total . .	79/160 (49 4 per cent.)	7/40 (17·5 per cent.)	0 01
2	10 12	28/40 24/40		
	Total . .	52/80 (65 per cent)	11/40 (27 5 per cent.)	0 01
3	20 24	19/40 17/40		
	Total . .	36/80 (45 per cent.)	15/40 (37·5 per cent.)	

Because of the variation in the death rate of the control animals in the three experiments recorded in table VII, a single experiment was later repeated under exactly the same conditions regarding mode of infection, treatment and so forth. Groups of animals from the same batch were infected and treated at different times after infection. The following results were obtained :—

> Treatment started 4 *hours* after infection—28/39 (72 per cent.) survived.
> Treatment started 8 *hours* after infection—21/39 (54 per cent.) survived.
> Treatment started 24 *hours* after infection—26/80 (32 per cent.) survived.
> No treatment—7/40 (17 per cent.) survived.

A similar series of experiments was carried out with mice infected with capsulated anthrax bacilli. Cultures were grown in specially aerated CCY medium and harvested at a time when smears showed that the organisms were fully capsulated (Gladstone, 1945, personal communication). The suspension was centrifuged and re-suspended in phosphate buffer at pH 7·6 and adjusted to opacity 1 on Brown's

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* Dr H. M. B Adam kindly examined the figures for all experiments statistically, calculating the probabilities and testing for homogeneity.

scale. This was immediately diluted 10,000 times and 0·1 ml. of this suspension injected intraperitoneally into mice. The injections were made as soon as possible after preparing the suspension, as the organism undergoes rapid lysis. Several colony counts were made of suspensions prepared in this way at different times and the results were remarkably consistent. The estimated number of organisms injected into the mice was about 500. Since this number is based on colony counts and the organism grows in chains, the actual number of bacteria injected was in all probability several times greater, and thus of the same order as the number of spores injected. Penicillin was administered in the same manner and three doses of 100 units were given at two-hourly intervals. The results are given in table VIII.

TABLE VIII

Infection of mice with capsulated anthrax bacilli : effect of treatment with penicillin started at different times after infection

Probable dose of organisms : 1000-2000

Dose of penicillin : 300 units in three doses of 100 units (equivalent human dose : 1,050,000 units)

Interval (hours) between infection and start of treatment	Survivors on 9th day	
	Treated	Controls
2	36/40 (90 per cent.)	0/40 (0)
4	157/200 (78·5 per cent.)	13/200 (6·5 per cent.)
8	21/40 (52·5 per cent.)	4/40 (10·0 per cent.)
12	34/80 (42·5 per cent.)	11/80 (14·0 per cent.)
16	12/30 (40·0 per cent.)	7/40 (17·5 per cent.)

These results show that treatment of vegetative infections with penicillin is effective even when its start is delayed 16 hours. On the other hand it is easy to discern the importance of early treatment by examining the mortality figures among the different groups of treated mice. The percentage of survivors falls sharply in the group whose treatment was delayed for 8 hours compared with the group treated after 4 hours.

The need for these large doses of penicillin was shown in a single experiment where different amounts were given at 4, 6 and 8 hours after infection (table IX).

TABLE IX

Infection of mice with capsulated anthrax bacilli : effect of different doses of penicillin given during the first 8 hours of infection

Dosage	Survivors on 9th day
3 doses of 25 units	9/40
3 " " 50 "	18/40
3 " " 100 "	25/40
Controls	0/40

Although these large doses of penicillin were necessary to deal effectively with a small initial infecting dose of vegetative organisms, a further experiment (table X) showed that the same amount of penicillin could also control infection initiated with 10–100 times this number of organisms.

TABLE X

Infection of mice with capsulated anthrax bacilli : effect of treatment with 300 units of penicillin in the first 8 hours with different infecting doses

Infecting dose	Suspension opacity	Penicillin	Survivors on 6th day
0·1 ml.	0·1	100 units 4th, 6th and 8th hour	0/20
0·1 „	0·01	100 „ „ „ „ „ „	10/20
0·1 „	0·001	100 „ „ „ „ „ „	16/20
0·1 „	0·0001	100 „ „ „ „ „ „	15/20
0·1 „	0·0001	No treatment	3/20

Discussion

The ability of *B. anthracis* to secrete penicillinase doubtless explains why large initial concentrations of spores are able to grow in solutions of penicillin that inhibit the growth of smaller numbers of spores. Exactly how this is brought about is not clear. Chain and Duthie (1944) have shown that suspensions of a sensitive staphylococcus of 1×10^7 organisms per ml. will continue to show some metabolic activity in the presence of concentrations of penicillin of 0·01 unit per ml. In the case of *B. anthracis* some activity is presumably possible when the organisms number 1×10^6 per ml. in the presence of as much as 1 unit of penicillin per ml. Even in concentrations of penicillin which will finally kill all the organisms originally present as spores, it has been shown that the process of germination is not interrupted by penicillin. Also it can be shown that large concentrations of spores (3×10^8 per ml.) in saline do not themselves inactivate penicillin. Under certain conditions (low penicillin concentration and high spore concentration) germination presumably takes place and limited metabolic activity of the resulting vegetative forms produces enough penicillinase to destroy the penicillin and allow free growth of the remaining bacteria.

These findings *in vitro* explain to a large extent the failure of penicillin as a therapeutic agent for artificial anthrax infections in rabbits and mice. Only with the vegetative form and when treatment was started early were the results of treatment at all striking (table VII). At this early stage of the infection, penicillin is capable of destroying ten or a hundred times the M.L.D. of vegetative anthrax bacilli (table X). On the other hand when treatment is delayed a nidus of infection is presumably established and the production of penicillinase renders treatment less effective.

Similar experiments with animals infected with anthrax spores did not show these clear-cut differences and the results of early treatment

were not so striking, although the actual number of infecting organisms was of the same order in each case. The conditions which govern the germination of anthrax spores *in vivo* remain completely obscure, but it is reasonable to assume that some may germinate after the last of the penicillin has been excreted. It must be assumed that germination and subsequent multiplication of the vegetative forms so produced takes place in conditions especially favourable to the organism, under which small (but theoretically effective) doses of penicillin are not enough to eradicate the infection, as indicated by the results of treatment prolonged for 6 days (table II). Circumstances favourable to the spores probably arise to some extent by chance, because viable spores capable of producing virulent organisms may be isolated from the tissues of animals up to at least three weeks after the injection of sublethal doses.

Since most naturally occurring anthrax infections are spore infections, treatment with penicillin would probably need to be on a generous scale to be effective.

Treatment with a prolonged series of injections was equally disappointing. Started late in the disease, as in the first two rabbit experiments, the penicillin is presumably quickly destroyed by penicillinase from the vast numbers of the organism present in the body. In the mice treated by 4-hourly injections of 10 units of penicillin, the striking feature was the prolongation of life and the development of a local abdominal lesion shown by gross œdema. In these animals the penicillin was probably only enough to prevent or delay a general systemic invasion. On the other hand the local lesion developed continuously despite the penicillin. If the local skin lesion of a rabbit which has been given a subcutaneous injection of anthrax spores is ground up, the extract contains penicillinase. In the lesions of anthrax there is probably a local concentration of penicillinase capable of destroying any penicillin that diffuses into them from the blood stream.

In man the results of penicillin treatment of cutaneous anthrax infections have been more successful and there were no deaths in the largest series of cases so treated (Ellingson *et al.*, 1946, personal communication). The host displays a considerable degree of natural resistance to the infection as witnessed by the fact that the mortality among cases of malignant pustule is recorded as being of the order of 5-15 per cent. before the introduction of chemotherapy. In such cases the penicillin might prevent the septicæmia which is so often the prelude to death while the natural defences deal with the local lesion.

SUMMARY

Penicillin in sufficient doses, if given early enough, will prolong and sometimes save the lives of rabbits and mice experimentally infected with anthrax.

The drug is more effective against vegetative than against spore infections ; surprisingly large doses must be given very early in the infection.

Difficulty in controlling infection appears to depend on three factors : (1) production of penicillinase by *B. anthracis* ; (2) persistence of spores in the tissues and their germination after the blood-penicillin level has fallen ; and (3) poor natural resistance of rabbits and mice to anthrax infection.

REFERENCES

ABRAHAM, E. P., CHAIN, E., FLETCHER, C. M., GARDNER, A. D., HEATLEY, N. G., JENNINGS, M. A., AND FLOREY, H. W. 1941. *Lancet,* ii, 177.

CHAIN, E., AND DUTHIE, E. S. . 1945. *Ibid.,* i, 652.

DUTHIE, E. S. . . . . . . 1944. *Brit. J. Exp. Path.,* xxv, 96.

FOSTER, J. W., AND WOODRUFF, H. B. 1944. *J. Bact.,* xlvii, 43.

GARDNER, A. D. . . . . . 1945. *Lancet,* i, 658.

HEILMAN, F. R., AND HERRELL, W. E. 1944. *Proc. Mayo. Clin.,* xix, 492.

MILES, A. A., AND MISRA, S. S. . 1938. *J. Hyg.,* Camb., xxxviii, 732.

MURPHY, F. D., LA BOCCETTA, A. C., AND LOCKWOOD, J. S. 1944. *J. Amer. Med. Assoc.,* cxxvi, 948.

STERNE, M. . . . . 1945. *S. African Med. J.,* xix, 208.

EFFECT OF SHOCK-PRODUCING SUBSTANCES ON EXPERIMENTAL ANTHRAX INFECTION IN MICE

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THIS work began with the question whether " shock " lowers resistance to bacterial infection. Many shock-producing injuries cause extensive tissue damage favourable to bacterial invasion ; but the point at issue was whether the state of shock itself enhanced the results of infection. From the experiments to be described it appeared that the lethal effect of anthrax spores for mice could be increased by a variety of substances which have been used for the production of experimental shock. With two of the substances tested there was evidence to suggest that enhancement of the infection might be brought about even if the test substance and spores were injected at different sites.

Hughes (1938) described a toxic substance in blister fluids from burns which lowered the resistance of rabbits to staphylococci and of mice to streptococci and pneumococci ; he found that normal human sera had no such effect. Hughes (1939) also reported that inflammatory exudates, but not passive transudates, enhanced the effect of experimental tuberculosis in guinea-pigs, shortening the survival time and giving rise to lesions whose histology suggested a more acute reaction than in the controls. Wilson, Macgregor and Stewart (1937-38) considered that a specific substance absorbed from the burned area was the probable cause of acute toxæmia in burns ; therefore, as a first step, it seemed worth re-investigating Hughes's reports of an infection-enhancing substance present in blister fluids but absent from normal sera. Later, other substances were also examined.

METHODS

General procedure. In every experiment at least three groups, each of 5 or 10 mice, were inoculated with : (1) test substance alone, (2) anthrax spores alone, and (3) test substance + anthrax spores. In some experiments a fourth control group was inoculated with spores + formalin, which invariably enhanced the lethal effect. Doses and routes of inoculation are described separately for each test substance. Although inoculated mice were observed for 30 days, only deaths up to 10 days were recorded as due to the experiment, because very few animals died later than this and their deaths did not seem likely to be connected with the test substances. The experiments were repeated as often as possible with each test substance that gave a suggestive result.

Infecting agent. Anthrax spores were chosen as the infecting agent because a suspension of spores, once prepared, would always be readily available and not subject to alterations in virulence as a result of repeated subculture. The anthrax strain Paddington VI (National Collection of Type Cultures *no. 100*) was used throughout. Spore suspensions were prepared by growing the organism on peptone-free agar plates for 10 days at 37° C., washing the resulting growth thrice in saline and heating at 60° C. for 30 minutes to destroy vegetative forms. A stock suspension was stored in the ice chest and 0·25 c.c. of a 1 : 100,000 dilution in saline was used for inoculation. Surface viable counts (Miles and Misra, 1938) showed that the actual number of spores inoculated ranged from 200-400 in different experiments. Mice that died were examined *post mortem* and the presence of anthrax infection was confirmed by the typical appearance of the spleen and by examination of films from the heart blood, peritoneum and spleen.

RESULTS

Anthrax spores alone

Twenty-two groups of 5 or 10 mice were injected intraperitoneally with 200-400 anthrax spores as described and a summary of the results in these control animals (table I) forms the best background against which to consider the results of inoculating different test substances at the same time as the spores.

TABLE I

Effect of anthrax spores alone (200-400 spores given intraperitoneally)

No. of mice killed/inoculated	No. of experiments	Total no. of mice killed/inoculated
0/5	3	0/15
1/5	6	6/30
2/5	8	16/40
2/10	3	6/30
3/10	2	6/20
Totals . .	22	34/135
Mortality 25·2 per cent.		

The earliest deaths came between 24 and 48 hours after inoculation and most fell between the second and fifth days. It will be seen that in 22 separate trials the number of mice killed by spores alone ranged from none out of five (three trials) to two out of five (8 trials) and that the death-rate for the whole group was 34 out of 135 (25·2 per cent.). Viable anthrax spores were found in the spleens of survivors killed 3 months after inoculation.

Blister fluid from burns

Through the kindness of Professor W. C. Wilson, we were able to examine blister fluid from five patients with burns. The fluid was

aspirated with sterile precautions from unbroken blisters resulting from burns sustained $5\frac{1}{2}$-48 hours before aspiration; the age of the burn did not seem to influence the result. The fluids were cultured aerobically and anaerobically for 4 days in blood broth at 37° C. and all proved sterile.

To test the effect of blister fluid on the anthrax-spore infection 0·25 c.c. of each fluid was mixed with 0·25 c.c. of saline containing the appropriate dilution of spore suspension and inoculated intraperitoneally into 5 mice at the same time as 10 control mice were injected intraperitoneally—5 with the same number of spores alone (200-400) and 5 with the same volume (0·25 c.c.) of the blister fluid under investigation. All inocula were made up to 0·5 c.c. by the addition of sterile saline where necessary.

All the 25 mice inoculated with the blister fluids alone remained well; the effect of spores alone has already been described. The results with spores + blister fluid (table II) showed that some, but

TABLE II

Effect of anthrax spores + blister fluid from burns
(intraperitoneal inoculation)

Fluid no	No of mice killed/inoculated
1	0/5
2	5/5
3	5/5
4	3/5
5	3/5

Five mice were inoculated with each of the fluids alone; all remained well. Effect of spores alone is shown in table I.

not all, blister fluids from human burns could enhance the lethal effect of the spores.

Normal human sera

The next step was to test serum from four healthy adult males between 20 and 30 years of age. The blood was drawn and the serum separated with sterile precautions, and the mouse inoculations were carried out exactly as described for blister fluid. All of 20 mice remained well that were inoculated with serum alone (five mice for each serum); the effect of spores alone has already been described. The results with spores + serum (table III) showed that some normal human sera could enhance the lethal effect of spores.

Human muscle

The observations on normal human sera suggested that it might be worth testing the effect of human muscle. Again through the help

of Professor W. C. Wilson, we were able to examine four pieces of human muscle (rectus abdominis) excised with sterile precautions from patients undergoing exploratory laparotomy. Immediately

TABLE III

Effect of anthrax spores + normal human sera
(intraperitoneal inoculation)

Serum no.	No. of mice killed/inoculated
1	5/5
2	3/5
3	3/5
4	4/5

Five mice were inoculated with each of the sera alone ; all remained well. Effect of spores alone is shown in table I.

after its excision the piece of muscle was taken to the laboratory, where it was weighed, cut up finely with scissors, and suspended by grinding it in sterile saline so that 0·25 c.c. of saline contained a known amount of muscle (0·075 g. in three experiments and 0·05 g. in one experiment). Inoculation of the mice was completed within $\frac{1}{2}$-1 hour of excision of the muscle. All samples were proved sterile by inoculating 10 c.c. of blood broth with 0·5 c.c. of the muscle suspension, incubating aerobically and anaerobically for 7 days at 37° C. and then plating out on blood agar.

For the tests, 0·25 c.c. of muscle suspension was mixed with 0·25 c.c. of saline containing the test dose of spores and inoculated intraperitoneally into 5 or 10 mice. At the same time equal numbers of control animals were inoculated with spores alone and muscle suspension alone. All of 25 mice remained well that were inoculated with muscle alone ; the effect of spores alone has already been described. The results with spores + muscle (table IV) showed

TABLE IV

Effect of anthrax spores + human muscle
(intraperitoneal inoculation)

| Experiment no. | " Dose " of muscle | No. of mice killed/inoculated | | |
		Spores alone	Muscle alone	Spores+muscle
1	0·075 g.	2/5	0/5	5/5
2	0·075 „	1/5	0/5	5/5
3	0·075 „	2/5	0/5	5/5
4	0·05 „	2/10	0/10	5/10

that in each of three experiments with 0·075 g. of muscle the lethal effect of the spores was enhanced. In the fourth experiment with

0·05 g. of muscle the difference between spores alone (2 out of 10 mice killed) and spores + muscle (5 out of 10 mice killed) was suggestive but not conclusive.

In experiments 1-3 the enhancing effect of the muscle inoculation on the lethal effect of the spores was not accompanied by any clinical signs of shock in the mice inoculated with muscle alone. Even when 0·3 g. of muscle was given intraperitoneally (5 mice) or subcutaneously (10 mice), the animals remained well throughout, and the enhancing effect was not evident when the muscle (0·075 g. and 0·3 g.) was given subcutaneously and the spores intraperitoneally.

Formalin

At the beginning of this work we recognised that our experiments resembled the early work on bacterial " aggressins " (reviewed by Browning, 1931). Our reading of this early work suggested the usefulness of including a known aggressin in each experiment; with this object it was our practice always to inoculate a group of five " positive controls " with the test dose of anthrax spores + 0·25 c.c. of peritoneal washings in 0·5 per cent. formol-saline of a mouse dead of anthrax. The formolised washings, which became sterile in the ice-chest, had no effect on the mice if injected alone, but invariably caused death of all 5 mice when inoculated intraperitoneally with the test dose of spores—a result which served as a useful control, especially when the spores alone failed to kill any of the 5 mice.

The results in this group were so regular that the matter seemed worth investigation. Accordingly we tried 0·5 per cent. formol-saline, using formalin of analytical-reagent purity, and found that this was nearly as certain in its effects (table V) as the formolised anthrax exudate.

TABLE V

Effect of anthrax spores + 0·5 per cent. formalin
(intraperitoneal inoculation)

Experiment no.	No. of mice killed/inoculated
1	4/5
2	5/5
3	5/5
4	5/5
5	5/5
6	4/5
7	10/10

In each experiment 5 or 10 mice were inoculated with 0·5 per cent. formalin alone; all remained well. Effect of spores alone is shown in table I.

The results of infection were not enhanced unless the formalin and spores were injected into the same site. In several such experiments,

0·25 c.c. of formol-saline in strengths of 0·5, 1, 2 and 5 per cent. formalin was injected subcutaneously and the usual dose of spores intraperitoneally. These procedures failed to increase the number of deaths from the spore infection. For about 10 minutes after inoculation the mice given 5 per cent. formol-saline showed symptoms of collapse and apparent discomfort at the site of injection, but they quickly recovered. None of the mice inoculated with 1 or 2 per cent. formalin had any visible reaction.

Miscellaneous substances

In four experiments, in the manner already described, each of the following substances was inoculated along with spores into the peritoneum of 5 mice : (1) tuberculous pus from a neck gland; (2) 0·5 per cent. phenol-saline ; (3) anti-anthrax serum with 0·4 per cent. tricresol ; and (4) normal rabbit serum. None of these substances increased or decreased the number of deaths from the spore infection.

Magnesium adenosine triphosphate

Through the kindness of Professor H. N. Green, we were given generous supplies from two freshly-prepared batches of magnesium adenosine triphosphate (Mg. A.T.P.),—the shock-producing substance identified in normal muscle by Green and his colleagues (Green, 1943, 1945 ; Bielschowsky and Green, 1943). The first batch had an actual Mg. A.T.P. concentration of 22·2 mg. per c.c., the second of 23 mg. per c.c.

TABLE VI

Effect of anthrax spores + magnesium adenosine triphosphate
(Mg. A.T.P.) in a dose of 3 mg. per 10 g.

Experiment no.	No. of mice (killed/inoculated)		
	Mg. A.T.P. alone	Spores alone	Mg. A.T.P.+spores
Mg. A.T.P. and spores together intraperitoneally			
1	1/8	3/10	9/10
2	0/10	2/10	9/10
Mg. A.T.P. subcutaneously, spores intraperitoneally			
3	0/10	2/10	13/20

Toxicity tests showed that intraperitoneal or subcutaneous inoculation of 3-5 mg. per 10 g. quickly induced severe collapse lasting 6-8 hours, and that with few exceptions all the mice thereafter

recovered. Accordingly we examined the effect of inoculating mice with the usual test dose of spores + 3 mg. per 10 g. of Mg. A.T.P. In two experiments the Mg. A.T.P. and spores were inoculated together into the peritoneal cavity; in a third the Mg. A.T.P. was given subcutaneously and the spores intraperitoneally. The results (table VI) showed that the intraperitoneal inoculation of spores + Mg. A.T.P. had a more lethal effect on the mice than the injection of either alone. The single animal killed by Mg. A.T.P. alone died in 12 hours; those killed by spores + Mg. A.T.P. died of anthrax between the second and fifth days, after they had fully recovered from the adenosine shock. With subcutaneous inoculation of Mg. A.T.P. and intraperitoneal inoculation of spores, the difference between the test and control groups was less marked but enough to be regarded as suggestive evidence that the shock induced by Mg. A.T.P. was accompanied by some reduction in resistance to the spore infection.

Hypertonic glucose (dehydration shock)

Cameron, Burgess and Trenwith (1946) recently described in detail the changes in rabbits and goats injected subcutaneously with large amounts of hypertonic glucose or sodium chloride solutions. They chose these solutions in order to study (p. 213) " some of the effects of rapidly progressive anhydræmia produced in such a way as to eliminate local tissue damage and the tendency to infection, complications which obscure the late burn picture ". Since our object was to test whether the tendency to infection was an essential part of the shock syndrome, we decided to test the effect of spores in mice which had been injected with hypertonic glucose to produce dehydration shock.

We chose the same dosage as Cameron *et al.* used for their experiments, namely 20 c.c. per kg. (0·2 c.c. per 10 g.) of 60 per cent. glucose solution; the glucose was of analytical-reagent purity and the solutions were made up in normal saline and sterilised by steaming for 20 minutes on 3 successive days. Mice were inoculated as already described with hypertonic glucose and spores, both intraperitoneally, and with hypertonic glucose subcutaneously and spores intraperitoneally. Given by either route, the hypertonic glucose rapidly induced a state of collapse which lasted 6-8 hours and was indistinguishable clinically from that resulting from magnesium adenosine triphosphate; none of the mice died that received hypertonic glucose alone. The results with spores and hypertonic glucose (table VII) were similar to those with spores and magnesium adenosine triphosphate. Hypertonic glucose and spores intraperitoneally produced a distinct increase of the lethal effect of the spores. When the spores were injected intraperitoneally and the hypertonic glucose subcutaneously the difference between test and control groups provided suggestive but

not conclusive evidence that the shocked animals were less able to resist infection.

TABLE VII

Effect of anthrax spores+hypertonic (60 per cent.) glucose in a dose of 0·2 c.c. per 10 g.

Experiment no.	No. of mice (killed/inoculated)		
	Hypertonic glucose alone	Spores alone	Hypertonic glucose + spores
Hypertonic glucose and spores together intraperitoneally			
1	0/10	3/10	9/10
2	0/10	2/10	10/10
Hypertonic glucose subcutaneously, spores intraperitoneally			
3	0/10	3/10	6/10
4	0/10	2/10	6/10

DISCUSSION

What emerges from these experiments is the variety of agents capable of enhancing the lethal effect of an anthrax-spore infection in mice : some normal human sera, some blister fluids from burns, 0·5 per cent. formalin, human muscle, magnesium adenosine triphosphate, and hypertonic glucose. Since our work was designed to explore the possibility that the state of shock was itself favourable to infection it is of interest to note that experimental shock has been produced by formalin (Selye and Dosne, 1940) and by muscle (Green, 1943) as well as by magnesium adenosine triphosphate and hypertonic glucose, and that all four substances, if injected into the peritoneum along with anthrax spores, were very regular in their action. Thus, along with spores, muscle (0·075 g.) killed 15 out of 15 mice (table IV), 0·5 per cent. formalin killed 38 out of 40 mice (table V), magnesium adenosine triphosphate killed 18 out of 20 mice (table VI), and hypertonic glucose killed 19 out of 20 mice (table VII)—a total of 90 out of 95 mice compared with 34 out of 135 mice killed by spores alone (table I). Muscle and formalin enhanced the infection in doses too small to produce any visible effect on the general condition of the animals, whereas magnesium adenosine triphosphate and hypertonic glucose rapidly caused severe collapse lasting 6-8 hours. When these two substances were each injected subcutaneously, and the spores intraperitoneally, the difference in mortality between test and control mice was suggestive rather than conclusive evidence that the infection had been enhanced (tables VI and VII) ; none of the others influenced the infection unless injected intraperitoneally along with the spores.

Injury to the peritoneal lining might account for the results with 0·5 per cent. formalin but damage to tissue defences at the site of inoculation does not explain all our observations. We publish our findings because they appear to support the view that shock-inducing substances can enhance the results of infection.

SUMMARY

It has been shown that a number of substances used for the production of experimental shock can enhance the lethal effect of anthrax spores for mice.   '

REFERENCES

BIELSCHOWSKY, M., AND GREEN, H. N.   1943. *Lancet*, ii, 153.

BROWNING, C. H.   1931. Medical Research Council, A system of bacteriology, *London*, vol. vi, p. 290.

CAMERON, G. R., BURGESS, F., AND TRENWITH, V.   1946. This *Journal*, lviii, 213.

GREEN, H. N.   1943. *Lancet*, ii, 147.

„   1945. *Brit. Med. Bull.*, iii, 102.

HUGHES, W. H.   1938. *Lancet*, ii, 670.

„   1939. This *Journal*, xlviii, 605.

MILES, A. A., AND MISRA, S. S.   1938. *J. Hyg.*, Camb., xxxviii, 732.

SELYE, H., AND DOSNE, C.   1940. *Lancet*, ii, 70.

WILSON, W. C., MACGREGOR, AGNES R., AND STEWART, C. P.   1937-38. *Brit. J. Surg.*, xxv, 826.

CARCINOMA OF THE PITUITARY GLAND WITH METASTASES TO THE LIVER IN A CASE OF CUSHING'S SYNDROME

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(PLATES XVIII AND XIX)

THIS case is considered worthy of publication on account of the extreme rarity of the condition and because of certain unusual features which emerged during the study of the lesions.

CASE REPORT

Abstract of clinical examination

The patient was a spinster of 43 on her first admission to hospital on 12 4 44 She complained of swollen ankles, growth of hair on the face of 8 months' and pigmentation of skin of 3 or 4 years' duration, and increase in weight during the past 1½ years. When examined, the skin had an intense dusky-red colour, B.P. was 230/130, Hb. 110 per cent. and red cells 5 5 million. X ray examination of the skull showed a normal pituitary fossa. The urine presented no signs indicative of adrenal tumour.

She was re admitted to the care of Mr N. M. Dott on 18.11.44 having obtained no relief of her previous symptoms : B P. was 200/134. Mr Dott considered her to be a typical example of Cushing's syndrome and advised a course of deep X-ray therapy to the pituitary. Little change was noticed as a result of this, and she was sent home on 22 12.44.

She was seen at regular intervals until re admitted on 5.9.45. No marked change had occurred, except that the face and neck showed deeper pigmentation and facial hair had increased in amount. She was still regarded as a Cushing's syndrome, but the complete failure of X ray therapy raised the possibility of a suprarenal lesion being responsible for her condition. Basophil adenoma of the pituitary was finally favoured because : (1) hormone estimations in the urine provided evidence in support of this view ; (2) X-ray examination of the skull showed erosion of the dorsum sellæ, though little if any enlargement of the pituitary fossa Mr Dott decided that further irradiation of the pituitary was indicated and implanted three radon seeds into the exposed organ. At operation, the pituitary was found to be normal on external examination. A few fragments of tissue were removed from the gland and hæmostasis was ensured by placing some fibrin foam in the little cavity thus produced. The material removed was examined by Colonel W. F. Harvey, who reported that it showed only necrotic tissue with an indistinct and largely acellular pituitary pattern and little or no leucocytic reaction. The appearances suggested radionecrosis

The operative findings ruled out the presence of a large adenoma capable of distorting the pituitary, but it was considered that a small adenoma well inside the organ could still be present. The patient made a good recovery from the operation and was discharged on 8.10.45 for a 3 months' rest, after which she was to be re-admitted for further assessment, with possible exploration of the suprarenals.

She was re-admitted on 2.1.46 *complaining of breathlessness and swelling of her legs with increased skin pigmentation.* Professor D. M. Dunlop was asked to see her and decided that she was suffering from *congestive cardiac failure, with a focal brain-stem lesion of vasospastic origin due to an acute hypertensive attack.* He suggested *arrhenoblastoma of the ovary* as an alternative diagnosis to a pituitary or suprarenal lesion. She was transferred to the care of Professor Dunlop for medical treatment, but steadily deteriorated during the last month of her illness. She became very apathetic and her intellectual powers gradually declined. Bedsores developed which did not respond to penicillin, a terminal rise in temperature and pulse occurred, and she died on 13.2.46.

Post-mortem examination

The body was that of a well-developed, well-nourished, middle-aged woman. The face was puffy and cyanotic, and the entire skin surface was of a distinct brownish-coppery colour, especially over the upper part of the body. Well-marked development of hair was present on the chin and upper lip, while the pubic hair was of masculine distribution.

The *serous sacs* showed no significant changes apart from obliteration of the apices of both pleural sacs by old adhesions.

Endocrine system

Pituitary gland (fig. 1). The entire gland showed moderate enlargement, especially in the transverse and antero-posterior planes, with consequent moderate expansion of the sella turcica. Superiorly there protruded a slightly lobulated mass of tissue of fairly firm consistence and greyish-white colour, over which the optic chiasma was stretched though not grossly compressed. The gland was everywhere adherent to the walls of the pituitary fossa and some slight difficulty was encountered in removing it. The bone in intimate contact with the pituitary was soft and cut easily with the knife, suggesting infiltration of its substance. A vertical section through the longest (transverse) axis of the suprasellar protrusion revealed a fairly firm mass of tissue of greyish-white colour, below which was a circumscribed zone of crumbling necrotic material. No signs of normal pituitary tissue could be made out. Three radon seeds were found in the tissue and were removed.

Suprarenal glands. Both were hypertrophied, with scattered small adenomatous nodules in the cortices.

Thyroid gland. A single spheroidal cyst 1·5 cm. in diameter was present in the right lobe of the thyroid : it contained thin brown fluid.

CARCINOMA OF PITUITARY

FIG. 1.—Transverse section of entire pituitary neoplasm: suprasellar protrusion to right. H. and E. ×5.

FIG. 2.—Metastatic nodules in liver. Natural size.

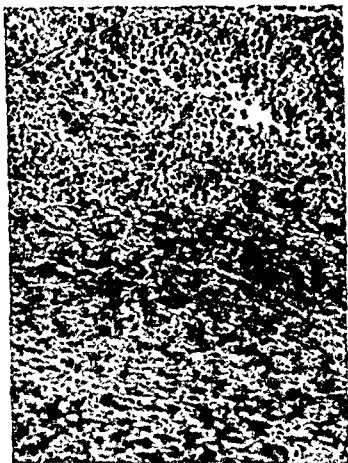FIG. 3.—Margin of pituitary neoplasm, showing infiltration of bone. H. and E. ×120.

FIG. 4.—Margin of metastatic neoplasm in liver: neoplasm above, liver below. H. and E. ×120.

Cardiovascular system

The *heart* was enlarged and its chambers moderately dilated, the left ventricle showing also well-marked hypertrophy. Apart from slight coronary atheroma, no other abnormalities were detected.

Digestive system

No abnormalities were found in any part of this system, with the exception of the liver.

The *liver* (fig. 2) was slightly swollen and soft in consistence, though retaining its usual shape. The parenchyma was pale yellowish-brown in colour, very soft and diffusely mottled. Scattered throughout the parenchyma at fairly wide intervals were eight spheroidal deposits of tumour tissue, ranging from 1·5 to 0·5 cm. in diameter. They were greyish white, firm and sharply demarcated from the surrounding liver parenchyma.

Respiratory system

Larynx, trachea and *bronchi*. The mucous membrane was congested and the lumen contained much muco-purulent secretion.

Lungs. Both apices showed fibrosis and the lower lobe of the left lung an almost completely confluent bronchopneumonia.

Genito-urinary system

Kidneys showed cloudy swelling and finely granular surfaces. The right pelvis contained a small calculus. Left pelvis normal, as were ureters and bladder.

Uterus and appendages normal.

Lymph vascular system

Lymph nodes were free from neoplasm.
Spleen showed septic changes.

Central nervous system

No abnormalities.

HISTOLOGICAL EXAMINATION

Pituitary gland (fig. 5). The entire gland is almost completely destroyed and replaced by a malignant neoplasm. Close to the periphery, in the superior part of the lesion, there is a small area of surviving normal anterior lobe, but apart from this, no signs of pituitary tissue can be found. With hæmatoxylin and eosin staining, the neoplasm is found to be composed of completely undifferentiated

cells packed closely together and exhibiting little pleomorphism. The majority of the cells are round or oval, except where mutual compression has caused some distortion, with a prominent nucleus and fairly abundant cytoplasm. The cytoplasm is everywhere light greyish pink in colour and appears to be free from granules. The nucleus is round and prominent and possesses a clear nuclear membrane. The chromatin takes the form of a very fine reticular network, with, in some cases, small scattered aggregations of chromatin material. A single bright-pink-staining nucleolus is a prominent feature in most of the nuclei, while a few show two nucleoli. Mitotic figures are scanty. Cell morphology is remarkably uniform, the majority of the cells conforming to the above description. In addition, scattered throughout the section at irregular intervals, there are distinct giant-cell types ranging in size from structures approximately twice as large as the uniform cells to bizarre types approximately four or five times as large. In the smaller types, the nucleus is merely an enlarged replica of the ordinary nucleus already described, whereas in the large giant cells the nucleus is either multiple or a single markedly convoluted structure with a relatively heavy chromatin content.

In the inferior part of the specimen, well below the projecting suprasellar nodule, there is an extensive area of necrosis, in some parts of which cell outlines can still be made out very indistinctly. In immediate relation to this area there is a small reticulated mass of fibrin, presumably the remains of the "fibrin foam" applied at the operation. At the lateral margins of the tumour, well-marked infiltration of the bony wall of the pituitary fossa can be seen (fig. 3).

The neoplastic cells are supported by a delicate fibrous stroma in which run fairly numerous small vessels composed of a single layer of endothelium. The neoplasm is composed of three distinct cell masses, namely the projecting suprasellar mass and two smaller masses below and lateral to this area. The three masses are separated by wide areas of fairly dense fibrous tissue, the structure of which contrasts strikingly with the delicate fibrous stroma running in the neoplastic tissue proper.

Sections were stained with eosin and pyrrol blue (Biggart, 1935) in order to study the condition of the cytoplasm (fig. 7). In most of the cells the cytoplasm is free from granules and stains uniformly greyish green, the general appearance greatly resembling the normal non-granular chromophobe cells of the anterior lobe. (Fig. 7 shows the cells bluer in colour than in the original section.) In addition, in two areas of the tumour there are small groups of cells whose cytoplasm contains distinct eosinophilic granules. This appearance is interesting in relation to the cytology of the cells found in the secondary deposits in the liver (vide infra). True basophilic cells are not present.

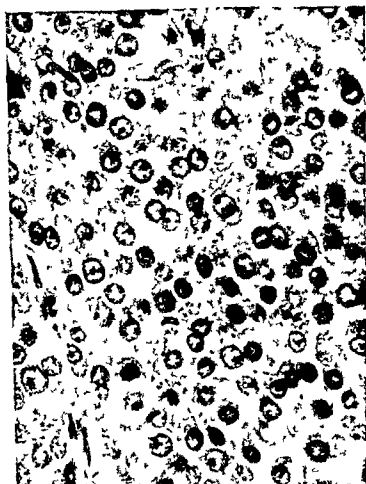In the small piece of surviving normal anterior lobe, the usual

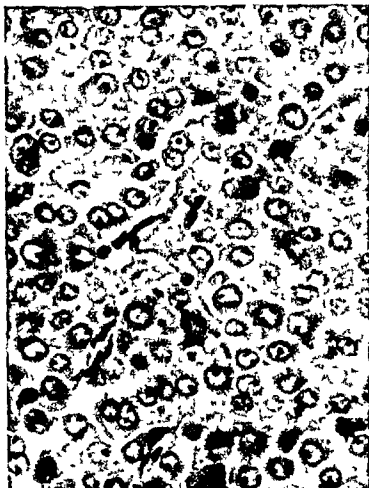CARCINOMA OF PITUITARY

FIG 5 —Pituitary neoplasm   H and E   ×600.

FIG 6 —Metastatic neoplasm in liver.   H and E.
×600.
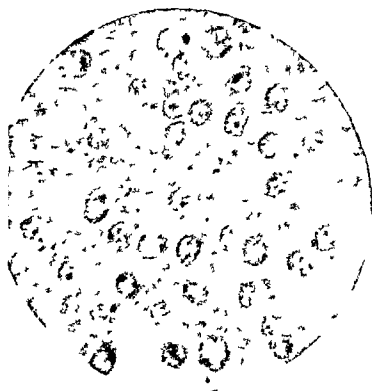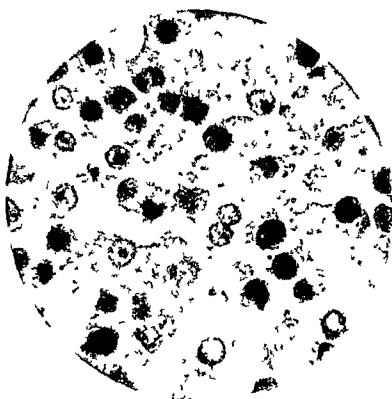

7 —Pituitary neoplasm   Eosin and pyrrol blue.
×850.

FIG. 8.—Metastatic neoplasm in liver   Eosin and
pyrrol blue.   ×850.

three cell types are well seen, and, in addition, a few of the basophilic cells show hyalinisation as described by Crooke (1935).

Liver (figs. 4 and 6). Examination of all the neoplastic deposits reveals a similar picture.

masses of completely stained with hæmatoxylin and eosin show that the morphology and general arrangement of the cells are identical with those of the pituitary neoplasm.

Sections stained with eosin and pyrrol blue, however, reveal a surprising picture (fig. 8). Almost without exception the cytoplasm of the various cells is granular, and in most of them the granules are closely packed and strongly eosinophilic in reaction. In the remainder the granules are purple, suggesting incomplete development of true eosinophilic granules. The appearance of the cells in general is very similar to that found in eosinophilic adenomata of the pituitary. Basophilic cells are not present and cells of chromophobe type similar to those seen in the pituitary neoplasm are either very scanty or apparently completely absent in some of the deposits.

Suprarenal glands. The cortex is increased in amount owing to the presence of fairly numerous areas of adenomatous hyperplasia and to hypertrophy of the cortical tissue in general.

Other organs show no significant changes apart from broncho-pneumonia in the left lung, mild renal arteriosclerosis, hypertrophy of the muscle fibres of the left ventricle and moderate septic changes in the spleen.

DISCUSSION

Primary carcinoma of the pituitary is a rare condition and its extra-cranial dissemination excessively rare. Ewing (1940) mentions very briefly a single case, quoted at second hand from an early paper which can safely be discounted. Bailey and Cutler (1940) surveyed the literature to 1940 and came to the conclusion that distant metastases had yet to be demonstrated. Dissemination within the cranial cavity or along the spinal meninges occurs occasionally and cases have been reported by Stolpe (1904), Cagnetto (1904), Smoler (1909) and Cairns and Russell (1931). Willis (1934) examined the few reported cases of extra-cranial dissemination as far as 1933 and concluded that most if not all of them were fallacious. Thus in some cases it appeared obvious that the neoplasm in the pituitary was itself a metastatic deposit from some extra-cranial source such as the lung, while in others the tumour was probably a carcinoma originating from pharyngeal epithelium and directly invading the pituitary, or a carcinoma originating from a residue of Rathke's pouch. He regarded the cases reported by Budde (1921) and Dott and Bailey (1925-26) as doubtful examples.

The case reported by Dott and Bailey can be deleted at once, since further examination of the material revealed the neoplasm in

the pituitary to be a metastasis from an extra-cranial source (personal communication from Mr N. M. Dott). The case reported by Budde is that of a spheroidal cell carcinoma of the pituitary with metastases to the cervical lymph nodes, lungs and pleura, but here again the evidence is inconclusive and the possibility of an origin from epithelial tissue other than that of the pituitary cannot be ruled out.

Thus as late as 1933 it would appear that no true and unreservedly acceptable example of a malignant pituitary neoplasm with extra-cranial metastases had been reported, and a thorough examination of the literature from 1934 to date has revealed only one acceptable example (that of Cohen and Dible, 1936). Following a search of the literature, these authors regarded their case as unique at that date. The patient was a spinster aged 50, who was diagnosed as a case of Cushing's syndrome of 5 years' standing. At post-mortem examination the pituitary appeared in the form of a dark, plum-coloured, lobulated tumour, which, on histological examination, had the structure of a basophilic carcinoma of the anterior lobe. The liver showed 3 or 4 small pale nodules of neoplastic tissue, the histology of which was similar to the lesion found in the pituitary. Increase in the amount of cortical tissue in both suprarenals was also present and the left ventricle of the heart was hypertrophied. The authors concluded that the case was one of Cushing's syndrome of 5 years' duration, presumably due to a basophil adenoma which, benign at the onset, had later changed to a basophil carcinoma.

The case presented in this paper is believed also to be an example of primary carcinoma of the pituitary with extra-cranial metastases. This assumption is based on the following observations. (1) The clinical evidence suggested that a lesion of the pituitary was responsible for the Cushing's syndrome presented by the patient. (2) Examination of all possible sites of neoplasia *post mortem* revealed neoplastic tissue only in the pituitary and liver. (3) Histological examination of the pituitary tumour showed it to be a chromophobe carcinoma of the anterior lobe, although a few cells containing eosinophilic granules were also present in some areas. (4) The nodules of neoplastic tissue in the liver faithfully reproduced the structure of the pituitary neoplasm, except for the presence of eosinophilic granules in the cytoplasm of most of the cells. (5) The cells in both neoplastic areas were quite unlike those found in primary hepatic carcinoma and the gross distribution of the hepatic lesions was also against this view. The possibility that the neoplasm in the pituitary was a metastasis from a primary hepatic carcinoma is so remote that it can be discounted.

Assuming that the neoplastic tissue in the liver was the result of dissemination from a primary carcinoma of the pituitary, the change in cell type from chromophobe to eosinophil requires some consideration. During the development of the normal chromophil cells of the anterior lobe of the pituitary it is believed by a number of

authorities that the chromophobe cells acquire granules and differentiate into one or other of the chromophil types (Severinghaus, 1938). Assuming that this view is correct, it is tentatively suggested that in the present case the primary neoplasm in the pituitary is a chromophobe carcinoma, while the secondary deposits in the liver have resulted from differentiation of the malignant chromophobe cells into eosinophilic cells of varying degrees of maturity. The presence of a few eosinophilic cells within the primary chromophobe tumour could also be explained as due to eosinophilic differentiation.

Since no signs of a malignant neoplasm of the pituitary were found at operation five months before death, it is reasonable to assume that the carcinoma found at autopsy was not responsible for the clinical manifestations which had developed several years before. The fact that the pituitary, when exposed at operation, was of normal appearance, does not, however, exclude the presence of a basophil adenoma of the anterior lobe, since these lesions are often quite small and lie well within the substance of the anterior lobe, producing little or no distortion of the gland (Biggart and Dott, 1936). Hence the suggestion is put forward that the development of a carcinoma in the pituitary was a relatively late phenomenon in this case, due to malignancy supervening in a small pre-existing basophil adenoma. The role, if any, played by X-radiation in this transformation is conjectural.

SUMMARY

A case of carcinoma of the pituitary with metastatic spread to the liver is described and discussed. The neoplasm was a late phenomenon in a case of Cushing's syndrome and was thought to have developed from a pre-existent small basophil adenoma of the anterior lobe of the pituitary. The cells comprising the pituitary neoplasm were predominantly of chromophobe type, while those in the metastases were almost all of eosinophil type. It is suggested that this appearance was due to incomplete differentiation of eosinophil cells from the malignant chromophobe cells.

REFERENCES

BAILEY, O. T., AND CUTLER, E. C.   1940.   *Arch. Path.*, xxix, 368.
BIGGART, J. H.   .   .   .   .   .   1935.   *Edinb. Med. J.*, xlii (*Trans. Edinb Obstr. Soc.*, 1934-35, xciv, 113).
BIGGART, J. H., AND DOTT, N. M.   1936.   *Brit. Med. J.*, ii, 1153 and 1206.
BUDDE, M.   .   .   .   .   .   .   1921.   *Frankf. Z. Path.*, xxv, 16.
CAIRNS, H., AND RUSSELL, D. S. .   1931.   *Brain*, liv, 377.
CAGNETTO, G.   .   .   .   .   .   1904.   *Arch. path. Anat.*, clxxvi, 115.

COHEN, H., AND DIBLE, J. H. . . . 1936. *Brain*, lix, 395.

CROOKE, A. C. . . . . 1935. This *Journal*, xli, 339.

DOTT, N. M., AND BAILEY, P. . . 1925-26. *Brit. J. Surg.*, xiii, 314.

EWING, J. . . . . 1940. Neoplastic diseases, 4th ed., *Phila-delphia* and *London*, p. 1017.

SEVERINGHAUS, A. E. . . . . . 1938. *Assoc. Res. Nerv. and Ment. Dis.*, Proc. (1936), xvii, 69.

SMOLER, F. . . . . 1909. *Wien. klin. Wschr.*, xxii, 1488.

STOLPE . . . . 1904. *Dtsch. med. Wschr.*, xxx, 686. (Abstr. of proceedings, 33rd Kongr. der Dtsch. Gesells. f. Chir., April, 1904).

WILLIS, R. A. . . . . . . 1934. The spread of tumours in the human body, *London*, p. 138.

HÆMOLYTIC ACTIVITY OF *C. DIPHTHERIÆ*

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HÆMOLYSIS by *C. diphtheriæ* is the subject of some conflicting reports. Earlier workers (Schwoner, 1904 ; Costa, Troisier and Dauvergne, 1918) found that whole broth cultures hæmolysed rabbit erythrocytes but that culture filtrates were inactive and the lytic activity was destroyed by heating. Hammerschmidt (1924) therefore concluded that there was no soluble hæmolysin and that hæmolysis was effected only by living bacteria. On the other hand Goldie (1933) claimed that hæmolysis by *C. diphtheriæ* was a property of cell-free filtrates, that hæmolysin production ran parallel with toxin production and that the hæmolysin was not destroyed by boiling.

Agreement is also lacking about the diagnostic significance of hæmolysis by strains of *C. diphtheriæ*. Costa *et al.* reported hæmolysis by all true diphtheria strains but not by diphtheroids, whereas Schwoner noted absence of hæmolysis with some true diphtheria strains, particularly from mild cases and in old cultures. Transience of hæmolytic activity was noted by Heeren and Megrail (1930). The separation of *C. diphtheriæ* into *mitis*, *gravis* and *intermedius* types has not clarified the position. Among *gravis* strains in Manchester Robinson (1934) found about one-third hæmolytic and half of these became non-hæmolytic on prolonged subculture. In Leeds both hæmolytic and non-hæmolytic *gravis* strains were found (Anderson, Happold, McLeod and Thomson, 1931 ; Robinson and Marshall, 1934). In Berlin, however, *gravis* strains were consistently hæmolytic (Christison, 1934-35 ; Schiff and Werber, 1935).

It seemed desirable, therefore, to collect more information about the hæmolytic activities of *C. diphtheriæ*, particularly as to the possible existence of a soluble hæmolysin.

EXPERIMENTAL OBSERVATIONS

Strains used. Two hundred and twenty-five strains were investigated, most of them recently isolated from patients, and others which had been kept in laboratory subculture for varying periods.

Observation of hæmolysis. Hæmolysis was observed in liquid cultures, since these are more amenable to quantitative study than cultures on solid media. The technique adopted as standard was the addition of 0·5 c.c. of a 5 per cent. suspension of washed rabbit erythrocytes to the culture or other preparation and adjustment of the total volume to 2 c.c. with saline. Tubes were incubated in a water-bath at 37° C. for one hour and the degree of hæmolysis was read immediately, since

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standing at room temperature had little effect on the result except to increase slightly degrees of partial hæmolysis. Under these conditions 1 c.c. of culture of an actively hæmolytic strain produced complete or nearly complete hæmolysis; there was less hæmolysis with 0·5 or 0·2 c.c. of culture and none with 0·1 c.c. Unless otherwise stated readings throughout this communication refer to the degree of hæmolysis produced by 1 c.c. of culture in 1 hour at 37° C.

Effect of age of culture. 0·5 c.c. of a rabbit red-cell suspension was added to 1 c.c. of culture of a *mitis* strain of *C. diphtheriæ* after varying incubation periods, and the degree of hæmolysis was observed after further incubation for one hour, with the results shown in table I.

TABLE I

Hæmolysis with C. diphtheriæ mitis *after varying periods of culture*

Incubation period of culture (hrs.)	0	1	2	3	4	18	48	72	96	144
Degree of hæmolysis	—	—	—	++	C	C	C	+++	—	~

C = complete or nearly complete hæmolysis.
+ + +, + +, etc. = varying degrees of partial hæmolysis.
— = no hæmolysis.

In general, with cultures of an active strain, maximum hæmolysis was observed after 3½-4 hours' incubation. These observations were made with a papain digest medium containing 0·3 per cent. maltose which was sterilised by filtration and steaming and seeded with a large inoculum (5 per cent. by volume of the medium). It is important to note that uniform media must be used for comparable results in hæmolytic tests.

Erythrocytes of different species. Nine recently isolated strains were grown for 18 hours in papain digest broth and the hæmolytic activities of the cultures were tested against suspensions of washed erythrocytes of five different species. Results are given in table II.

TABLE II

Hæmolysis with different strains of C. diphtheriæ *against erythrocytes of various animals*

Strain	5595	5596	7	5601	5641	5650	9	8	5638
Type	*mitis*				*gravis*			*intermedius*	
Rabbit cells	C	+++	C	±	±	—	—	—	—
Sheep cells	C	+++	+++	∓	∓	—	—	—	—
Guinea-pig cells	+++	∓	+++	∓	—	—	—	—	—
Human cells	++	+	+++	∓	∓	—	—	—	—
Horse cells	+++	+	++	—	—	—	—	—	—

Repeat experiments with different preparations of erythrocytes gave variations in detail but the same general results were obtained, and Goldie's observation of considerably greater sensitivity of guinea-pig erythrocytes was not repeated. Accordingly rabbit erythrocytes were used in most of the succeeding experiments since they are conveniently obtained and the results are readily comparable with those of other workers.

Different strains. Similar results were obtained with either liquid cultures or blood-agar plates. Definite hæmolysis was sometimes observed on plates after 18 hours' incubation, but on other occasions further incubation or standing at room temperature was necessary before hæmolytic zones became manifest. The hæmolytic effects produced by 1 c.c. of papain digest cultures of the strains examined are given in table III.

TABLE III

Hæmolysis with various strains of C. diphtheriæ

Type					No. of strains examined	Number		
						Strongly hæmolytic	Trace	None
mitis	.	.	.	.	120	77	10	33
gravis	.	.	.		80	32	15	33
intermedius	.	.			25	0	0	25

It will be noted that no *intermedius* strain showed any hæmolytic activity, but that among both *gravis* and *mitis* strains some were hæmolytic and others non-hæmolytic. In *mitis* strains there is some indication that hæmolytic activity can be correlated with antigenic structure. Investigations on the serological typing of *mitis* strains are not yet complete but results so far obtained are as follows. Of 42 strains belonging to 4 serological types all were hæmolytic, whilst of 11 strains belonging to another type none was hæmolytic. Nevertheless some *mitis* strains have been isolated which, although agglutinated by the same serum, differed in hæmolytic activity. There is some indication that these strains may be capable of differentiation by more detailed serological investigation.

By way of contrast the hæmolytic activity of *gravis* strains showed little correlation with serological type. Among 45 strains of type I which have been isolated there was every degree of hæmolytic activity from the strongly hæmolytic to the completely inactive; 21 strains were strongly hæmolytic, 8 showed a trace of hæmolysis and 16 gave no hæmolysis. With type II strains all are apparently hæmolytic when freshly isolated. Of 15 strains belonging to 8 other serological types none was strongly hæmolytic.

In most of the succeeding experiments one *mitis* strain (5596) was used and crucial experiments were repeated with other strains.

Bacterial suspensions. Bacterial suspensions of hæmolytic strains were prepared by washing off serum agar slopes or centrifuging broth cultures. For hæmolytic tests saline proved as effective a suspending medium as broth despite the lethal effect of saline in dilute suspensions reported by Holt and Wright (1940). All the suspensions were hæmolytic.

Disintegrated organisms. Bacteria washed off serum agar slopes and found to be actively hæmolytic were dried *in vacuo* and disintegrated partially by grinding in an agate ball mill for 48 hours. Some intact organisms were still visible in a stained smear but the suspensions (and sterile filtrates) were completely non-hæmolytic. Extracts of the ground organisms still fixed complement in the presence of homologous sera, indicating that the grinding process had not destroyed cell constituents.

Culture filtrates and toxins. Actively hæmolytic cultures in different media were incubated for periods ranging from 4 hours to 10 days and were then filtered through Ford filter pads of different porosity and through Maasen filters, but all the filtrates were inactive. In view of Goldie's claim to have obtained hæmolytic filtrates of *C. diphtheriæ* with hæmolytic activity proportionate to toxin content, and since my recently isolated strains were poor toxin producers compared with some laboratory strains, I investigated a toxigenic Park-Williams 8 strain. Again however the filtrates were all non-hæmolytic although some contained 80 Lf of toxin per c.c., whereas Goldie's most potent filtrate contained only 26 Lf per c.c. Different filtration technique did not affect the result. Various reagents were added to the cultures both before and after filtration, including iodoacetate, hydrogen peroxide, potassium ferricyanide, potassium cyanide, cysteine, thioglycollate and toluol—all with negative results.

A further possibility remained that the discrepancy between Goldie's reported observation of hæmolytic filtrate and the failure of other workers to obtain a soluble hæmolysin was due to differences in the technique of observing hæmolysis. As already stated, rabbit cells were used in the present experiments and hæmolysis was observed after one hour's incubation at 37° C., whereas Goldie used guinea-pig cells, incubated for 4 hours and observed hæmolysis after a further 18 hours at room temperature. Even following Goldie's technique exactly, however, no hæmolysis was observed in culture filtrates.

In an attempt to find an explanation of the discrepancy control experiments were set up. The *p*H of the filtrates examined in the present experiments did not exceed 8·2 or 8·4, but in some media more alkaline reactions were reached and frequently the *p*H rose with the potency of the toxin. Therefore samples of culture medium without toxin were adjusted to *p*H 9·0, 9·5 and 10·0 and these were incubated with erythrocytes as in Goldie's experiment. There was

little hæmolysis at pH 9·0, considerable hæmolysis at 9·5 and complete hæmolysis with broth at pH 10·0. It is possible therefore that the heat-stable hæmolysin of Goldie was due to the alkaline reaction of the culture filtrate.

Burnet's (1930) method of obtaining staphylococcus hæmolysin was applied to *C. diphtheriæ*. This involved cultivation in 0·5 per cent. agar in an atmosphere containing 80 per cent. O_2 and 20 per cent. CO_2. Hæmolytic tests of filtrates were negative with Burnet's method and when 10 per cent. of serum and 0·1 per cent. of potassium ferricyanide were added, either together or separately, in order to enhance growth.

Extraction of hæmolytic streptococci with serum (Weld, 1934, 1935) yields a hæmolysin but this technique applied to *C. diphtheriæ* failed to yield hæmolytic filtrates.

Dialysates. A cellophane bag of broth was inoculated with a hæmolytic strain of *C. diphtheriæ* and suspended in a vessel containing a 5 per cent. suspension of erythrocytes, and the whole incubated at 37° C. for 48 hours: no hæmolysis was observed.

Supernatant fluids. Since it is possible that any soluble hæmolysin produced by *C. diphtheriæ* might be adsorbed by filters, actively hæmolytic cultures were centrifuged but hæmolysis was not observed with any of the supernatant fluids, although all the variations described in the section on filtrates were investigated. The centrifuged bacteria when re-suspended were found to have the same hæmolytic activity as uncentrifuged cultures.

A variation of these experiments was made by adding erythrocytes to the culture before centrifuging. Under these conditions the organisms centrifuged down very rapidly. The mixture of bacteria and erythrocytes showed hæmolysis when re-suspended, but the supernatant fluids did not hæmolyse fresh erythrocytes. There was an apparent acceleration of hæmolysis when the bacteria and erythrocytes were incubated for a short time before centrifuging.

Effect of various agents on hæmolysis by C. *diphtheriæ*

SH compounds and reducing agents. Cysteine added to cultures immediately inhibited hæmolysis down to a dilution of 1:2000, but the oxidised form, cystine, had no effect even at 1:200. This inhibiting effect of cysteine was reversed by reagents which combine with SH groups, e.g. iodoacetic acid. The same immediate reversal of cysteine inhibition was observed regardless of the order in which the various reagents, i.e. culture, erythrocytes, cysteine and iodoacetate, were mixed. Reversal of the effects of cysteine was also obtained by oxidation with atmospheric oxygen when the cultures were gently shaken in air.

In another experiment cysteine was added to a hæmolytic culture followed by erythrocytes. There was no hæmolysis after an hour's incubation and the culture containing erythrocytes was centrifuged. The re-suspended mixture of bacteria and erythrocytes showed hæmolysis, whilst the supernatant fluid inhibited the hæmolysis of erythrocytes on a fresh culture. This experiment indicates that cysteine remains free in the culture fluid and is not anchored

to the bacteria or erythrocytes, although there is a possibility that a minute amount of cysteine may have been attached to the cells but was oxidised when they were re-suspended. Two other compounds containing SH groups, namely thioglycollic acid and sodium hydrosulphite, also inhibited diphtheria hæmo-lysis. On the other hand with thiourea and thiouracil, which do not possess SH groups but contain sulphur and inhibit some enzyme actions, no inhibition of hæmolysis was observed even at a concentration of 1 : 500.

The mild reducing agent, ascorbic acid, which is free from SH groups, did not inhibit hæmolysis in concentrations up to 1 : 50.

Oxidising agents. Various oxidising agents failed to inhibit hæmolysis and even appeared to have a slight accelerating effect, the oxidising agents in-vestigated being 0·5 per cent. of 20-volume hydrogen peroxide, 1 per cent. potassium ferricyanide and 0·1 per cent. sodium hypochlorite. Higher con-centrations of hydrogen peroxide (5 per cent. of 20-volume reagent) inhibited hæmolysis and growth of cultures. The accelerating effect of oxidising agents on hæmolysis may be due to the increased rate of multiplication under oxidising conditions. The increased multiplication rate is not necessarily reflected in the results of viable counts, which represent the net result of multiplication less death, and it is probable that oxidising agents accelerate the death rate of organisms as well as the rate of multiplication.

Methylene blue in a concentration of 0·0002 per cent. had no effect on hæmolysis but 0·002 per cent. had a slightly inhibiting effect and 0·02 per cent. methylene blue inhibited hæmolysis completely, probably by its antiseptic action.

Antiseptics and other chemicals. Treatment of cultures with toluene for 30 minutes partly inhibited hæmolysis and longer treatment suppressed hæmolysis completely. Hæmolysis was also inhibited by 0·00005 per cent. acriflavine, 0·0001 per cent. thiomersalate (merthiolate), 0·02 per cent. iodine, 0·5 per cent. phenol, 0·5 per cent. formalin, and 5 per cent. hydrogen peroxide (20-volume reagent), but 0·2 per cent. iron alum had little effect. Hæmolysis was unaffected by 4 mg. of copper per c.c. (added as copper sulphate) but was completely inhibited by 10 mg. per c.c., which also inhibited multiplication of the bacteria. No effect on hæmolysis was produced by 0·2 per cent. hydro-quinine or anthraquinone sulphonate, or by 1 per cent. glucose.

With iodoacetic acid (sodium salt), 0·1 per cent. or less had no effect on the hæmolytic activity of 4-hour broth cultures, but 0·2 per cent. was slightly inhibitory and 0·5 per cent. iodoacetate suppressed hæmolysis completely. Added to a culture at the beginning of incubation, 0·02 per cent. or more of iodoacetate had a slight bacteriostatic effect, and both growth and hæmolytic activity were diminished. Potassium cyanide (0·05 per cent.) had no effect on hæmolysis but in high concentrations inhibition of hæmolysis was observed.

Streptolysin is inhibited by cholesterol suspensions at a dilution of one in a million (Hewitt and Todd, 1939) but cholesterol had no effect on hæmolysis by *C. diphtheriæ* even at 1 : 1000. Hæmolysis by the α-toxin of *Cl. welchii* is exalted when the calcium-ion content is increased, but neither addition of calcium nor decrease of calcium ions by addition of oxalate affected hæmolysis by *C. diphtheriæ*.

Heat resistance. Hæmolysis was entirely inhibited when cultures were heated in a water-bath for 5 minutes at 52° C., or for 15 minutes at 46° C. and was retarded even by 15 minutes at 43° C.

Hydrogen ion concentration. With adjustment of pH, cultures showed optimum hæmolysis at pH 7 and 8 and inhibition at pH 6 and 9 (table IV).

Normal serum. Hæmolysis was inhibited by the addition to broth of 10 per cent. or more of rabbit serum. The effect of normal horse serum is shown in table V. Cultures were grown in broth containing various amounts of normal horse serum, and the bacteria were centrifuged down and re-suspended in

broth containing the same amount of serum. The suspensions and supernatants were then tested for hæmolytic activity.

TABLE IV

Effect of pH on hæmolysis by C. diphtheriæ

pH	6·0	7·0	8·0	9·0
Hæmolysis	—	C	C	+++

TABLE V

Hæmolysis in presence of normal horse serum

Amount of serum in broth (per cent.)	Suspension	Supernatant fluid
2	C	—
5	C	—
10	+++	—
20	++	—

Antitoxic and antibacterial sera. Potent refined and concentrated antitoxic sera were diluted 1 : 100 in order to avoid the effects of serum proteins and 0·25 c.c. of antitoxin dilution was added to 1 c.c. of culture (table VI).

TABLE VI

Effect of antitoxin on hæmolysis

Antitoxin added	Hæmolysis
nil	C
Diphtheria, 10 units	C
Welch, 4 units	C

Since diphtheria antitoxin had no effect on hæmolysis an antibacterial serum was prepared in rabbits. The serum, which agglutinated the homologous strain completely at a dilution of 1 : 250 in 1 hour at 55° C., had no effect on hæmolysis when it was added to the cultures at a dilution of 1 : 25 and the mixture incubated for an hour at 37° C. before the addition of erythrocytes.

Penicillin. Penicillin inhibited hæmolysis, the inhibition being parallel with the effect on growth and more rapid in younger cultures. The effect of adding penicillin to cultures of various ages and incubating for various times before adding erythrocytes to test for hæmolysis is summarised in table VII.

Shaking the penicillin-treated culture accelerated inhibition. Without shaking, an 18-hour culture had to be incubated with

penicillin for some 5 hours to inhibit hæmolysis but with continuous gentle agitation complete inhibition of hæmolysis was observed after

TABLE VII

Effect of 10 units of penicillin on hæmolysis

Time of incubation with penicillin (hrs.)	Age of culture (hrs.)		
	3½	5	18
0	±	C	C
½	—	+++	C
1	—	∓	C
2	—	—	C
3	—	—	+++
5	—	—	+

half-an-hour's incubation, although the hæmolysis of cultures without penicillin was unimpaired by shaking. It is not certain whether the accelerated action of penicillin in this experiment was due to breaking down of clumps of bacteria, thus exposing them more rapidly to penicillin attack, or whether the resulting aeration accelerated growth, since an increased rate of multiplication may lead to accelerated bactericidal action by penicillin. As shown later, the technical difficulties in viable counts of *C. diphtheriæ* were too great for accurate measurement.

In experiments not yet completed it has been found that increased resistance to penicillin may follow subculturing *C. diphtheriæ* in broth containing penicillin. The hæmolytic activity of four strains originally hæmolytic was reduced after some 40 subcultures in penicillin broth.

TABLE VIII

Hæmolytic activity of penicillin-resistant C. diphtheriæ

Strain	Type	Hæmolytic activity	
		Original strain	Penicillin-resistant
5595	*mitis*	C	+++
5596	,,	C	+++
5601	*gravis*	∓	—
5641	,,	∓	—

Viable counts. Attempts were made to correlate the hæmolytic activity of cultures with their rate of multiplication. Direct counts in smears or emulsions were most inaccurate owing to the formation of clumps of bacteria, and the same difficulty was encountered in attempting viable counts with plating-out methods. The bacteria adhered to the walls of pipettes, making dilutions inaccurate, and pouring the dilutions on to the surface of plates was valueless, since the organisms collected in a few large clumps. Some improvement was obtained by mixing the dilutions with melted agar before growing the plates, but the oxygen requirements of *C. diphtheriæ* led to some failure of growth, particularly in agar recently de-aerated by steaming. This difficulty was

overcome by incorporating an oxidising agent—potassium ferricyanide—in serum agar. This medium appeared very suitable for the cultivation of *C. diphtheriæ* in deep agar plates and was made up as follows : 100 c.c. of 2 per cent. nutrient agar was melted and cooled to 55° C. ; then 10 c.c. of horse serum were added, followed by 1 c.c. of a 10 per cent. solution of potassium ferricyanide freshly made up and filtered through a Ford pad. Counts were still liable to gross inaccuracies and, although some improvement was effected by shaking the bacterial suspensions with glass beads to break up clumps, the results remained unreliable, as Wilson (1935) and Holt and Wright (1940) found with other bacteria.

Corynebacterium pyogenes *hæmolysin*

C. pyogenes forms a soluble hæmolysin (Lovell, 1937) and lysin was prepared for comparison with that of *C. diphtheriæ*. The filtrate from a 48-hour meat-broth culture was lytic at a dilution of 1 : 200 and was neutralised by the serum of a horse immunised with the lysin, but not by diphtheria antitoxin.

Unlike the findings with *C. diphtheriæ*, lysis was unaffected by the addition of cysteine. On the other hand hæmolysis by *C. pyogenes* lysin was inhibited by cholesterol in suspension at a dilution of one in a million, whereas *C. diphtheriæ* hæmolysis was not inhibited by 1 : 1000 cholesterol. Hæmolysis by the two species of corynebacteria is thus entirely different. *C. ovis*, however, resembles *C. diphtheriæ* in not yielding a soluble hæmolysin (Carne, 1939).

DISCUSSION

For diagnostic purposes, hæmolysis by *C. diphtheriæ* is of doubtful significance. Among 200 recently isolated strains examined, about two-thirds of the *mitis* strains exerted varying degrees of hæmolytic activity and about one-half of the *gravis* strains, but no *intermedius* strain produced any hæmolysis. The varying results in the literature are probably due partly to divergencies in technique, since differences in culture media, cultural conditions and method of observing hæmolysis yield contradictory results, and partly to the occurrence of hæmolytic and non-hæmolytic strains in epidemics in different localities. The strains in this investigation belonged to many different serological types but the typing is not yet completed. There was an apparent correlation between degree of hæmolysis and serological type with *mitis* but not with *gravis* strains. It is hoped to elucidate this point in further work, but it may be significant that, of 10 *mitis* strains kept in sub-culture and then dried, 8 were actively hæmolytic three years later, whereas of 10 *gravis* strains kept under the same conditions none was hæmolytic.

The recognition of *intermedius* types presented little difficulty using colonial appearance and microscopic examination of stained preparations, although an occasional *intermedius* strain may be wrongly reported. The lack of hæmolytic activity in *intermedius* strains is therefore not a valuable aid in diagnosis. The colonial

appearances on chocolate-tellurite-agar plates of many *mitis* and *gravis* types are however quite atypical but here again hæmolytic activity is of little value for differentiation, since both types may be either hæmolytic or non-hæmolytic. Starch fermentation must remain a criterion for differentiation, but even the results of this test may not be free from ambiguity as shown in the case of one strain, which when received was reported as being of *mitis* type but was found to ferment starch and to be agglutinated by a rabbit serum prepared against an authentic *gravis* strain. On plating-out, however, several colonies were selected, some of which proved to be starch fermenters and were agglutinated by one of the *gravis* type sera ; the other colonies were of *mitis* type and did not ferment starch but were agglutinated by one of the *mitis* type sera. In considering the results of type differentiation tests, therefore, due weight should be given to the possibility of infection with more than one type.

With hæmolytic strains of *C. diphtheriæ*, both cultures and suspensions of living bacteria are hæmolytic but all filtrates examined have been devoid of hæmolytic activity. Living organisms, therefore, seem necessary for hæmolysis and no evidence of a soluble hæmolysin has been found. This is in accord with the experience of other workers with the exception of Goldie, who claims that diphtheria toxin filtrates contain a soluble hæmolysin, the hæmolytic activity of which runs parallel with the toxin potency. This has not been confirmed, since toxin filtrates with even three times the potency of Goldie's most potent toxin were completely devoid of hæmolytic activity. It is perhaps significant that Goldie's hæmolysin was not destroyed by boiling—an observation highly suggestive of a chemical artefact. No *p*H measurements are recorded by Goldie but it is well known that diphtheria cultures in some media develop a highly alkaline reaction due to the oxidation of fatty acids to carbonates (Hewitt, 1930). Furthermore, toxin is formed in the later stages of growth when alkali formation is also at its height, so that the most potent toxins, which Goldie found the most hæmolytic, are often the most alkaline. Using Goldie's technique of prolonged incubation for the observation of hæmolysis, I found that adjustment of broth without toxin to *p*H 9·5 or 10 yielded an artificial " hæmolysin " which was not destroyed by boiling.

It is difficult, however, to exclude the possibility that a soluble hæmolysin may be formed by *C. diphtheriæ* under some hitherto undefined cultural conditions, since all evidence must necessarily be of a negative nature. The one possible piece of evidence making it worth while to continue the search for a soluble lysin was the zone of hæmolysis surrounding colonies on a blood agar plate. Although this zone is generally only a fraction of a millimetre wide it is difficult to visualise how the bacteria can lyse erythrocytes with which they are not in actual physical contact except through the agency of a soluble lysin.

In order to account for the lack of success in obtaining a soluble hæmolysin the possibility was considered that *C. diphtheriæ* produces not only a hæmolysin but also an enzyme system capable of inactivating the lysin in filtrates. It might be possible, therefore, to inactivate the destructive enzyme and thus allow the hæmolytic activity to become manifest. The presence of sulphydryl groups is necessary for many proteolytic enzymes and other disruptive systems to function, but sulphydryl groups are not only unnecessary for hæmolysis by *C. diphtheriæ*: their presence actually inhibits hæmolysis—an inhibition perhaps due to activation of disruptive enzymes. Therefore, a reagent which destroys SH groups but does not interfere with hæmolysis should inactivate the disruptive enzyme and thus protect the hæmolysin, which would then appear in culture filtrates. Several such SH-inactivating reagents have been found, each without effect on the hæmolytic activity of diphtheria cultures except in high concentration; they are iodoacetic acid, cyanide and hydrogen peroxide. But with these reagents I never found evidence of a hæmolytically active filtrate. Filtration of cultures was carried out under a variety of conditions, but it was still possible that the hæmolysin had been adsorbed by the filters. This possibility was excluded, however, by centrifuging the cultures. The supernatant fluids were devoid of any hæmolytic activity although the re-suspended deposit of organisms was as strongly hæmolytic as the original culture, showing that centrifugation was not destructive of the hæmolysin.

Only living cultures of *C. diphtheriæ* have been found to be hæmolytic and hæmolysis is inhibited by any treatment likely to stop active multiplication. Thus the common antiseptics immediately inhibit hæmolysis, as does heating for 15 minutes at 46° C. *C. diphtheriæ* multiplies actively when the oxygen supply is abundant, and oxidising agents such as ferricyanide, peroxide and hypochlorite, which inhibit soluble hæmolysins such as streptolysin, are without effect on *C. diphtheriæ* except in high concentrations, which are antiseptic. On the other hand cysteine and other SH compounds are inhibitory even in low concentrations, but the oxidised form cystine is without effect. Reagents such as iodoacetate which destroy SH groups reverse the inhibitory effect of cysteine. The reducing agent ascorbic acid does not inhibit hæmolysis, which suggests that the inhibitory effect of SH compounds may not be due to their reducing activity. On the other hand no specific combination of the cells with cysteine could be demonstrated.

The dependence of hæmolysis on the presence of actively multiplying organisms is confirmed by the action of penicillin. Young cultures in the logarithmic phase of growth, which are rapidly killed by penicillin, show a rapid disappearance of hæmolysis when penicillin is added, but with older cultures, which are less sensitive to penicillin, the inhibition of hæmolysis by penicillin is also delayed by several hours.

Several respects have been mentioned in which hæmolysis by *C. diphtheriæ* cultures differs from that produced by various soluble hæmolysins of bacterial origin. Another example is the effect of cholesterol, which has no appreciable effect on hæmolysis by diphtheria cultures but which inhibits hæmolysis, not only by streptolysin, pneumolysin and the θ-lysin of *Cl. welchii*, but also by the soluble hæmolysin of another Corynebacterium, *C. pyogenes*.

SUMMARY

1. Hæmolysis by *C. diphtheriæ* was observed only in the presence of living bacteria.

2. Any treatment interfering with bacterial multiplication inhibited hæmolysis.

3. Culture filtrates, extracts and supernatant fluids were all devoid of hæmolytic activity.

4. No evidence could be found for the existence of a soluble hæmolysin.

5. Penicillin inhibited hæmolysis, rapidly in young actively multiplying cultures and slowly in older cultures.

6. Strains in which penicillin resistance was developed showed decreased hæmolytic activity.

7. Cysteine and other SH compounds inhibited hæmolysis.

8. Some two-thirds of recently isolated *mitis* strains of *C. diphtheriæ* and about half the *gravis* strains possessed some degree of hæmolytic activity, but no *intermedius* strain was hæmolytic.

9. There was some evidence of correlation between hæmolytic activity and serological type with *mitis* but not with *gravis* strains. Subculture of *gravis* strains led to diminution in hæmolytic activity but this was not observed with *mitis* strains.

10. Difficulties in the differentiation of some *gravis* and *mitis* types are discussed.

REFERENCES

ANDERSON, J. S., HAPPOLD, F. C., McLEOD, J. W., AND THOMSON, J. G. 1931. This *Journal*, xxxiv, 667.

BURNET, F. M. . . . . 1930. This *Journal*, xxxiii, 1.

CARNE, H. R. . . . . 1939. This *Journal*, xlix, 313.

CHRISTISON, M. H. . . . . . 1934-35. *Zbl. Bakt.*, Abt. I, Orig., cxxxiii, 59.

COSTA, S., TROISIER, J., AND DAUVERGNE, J. 1918. *Compt. rend. Soc. biol.*, lxxxi, 89.

GOLDIE, H. . . . . 1933. *Ibid.*, cxii, 1210.

HAMMERSCHMIDT, J. . . . . 1924. *Zbl. Bakt.*, Abt. I, Orig., xciii, 413.

HEEREN, R. H., AND MEGRAIL, E. 1930. *J. Inf. Dis.*, xlvi, 485.

HEWITT, L. F. . . . . . . 1930. *Biochem. J.*, xxiv, 669.

HEWITT, L. F., AND TODD, E. W. 1939. This *Journal*, xlix, 45.

HOLT, H. D., AND WRIGHT, H. D. 1940. This *Journal*, li, 287.

LOVELL, R. . . . . . . 1937. This *Journal*, xlv, 339.

ROBINSON, D. T. . . . . . 1934. This *Journal*, xxxix, 551.

ROBINSON, D. T., AND MARSHALL, 1934. This *Journal*, xxxviii, 73.
F. N.

SCHIFF, F., AND WERBER, M. . . 1935. *Dtsch. med. Wschr.*, lxi, 259.

SCHWONER, J. . . . . . . 1904. *Cbl. Bakt.* Abt. I, Orig., xxxv, 608.

WELD, J. T. . . . . . . 1934. *J. Exp. Med.*, lix, 83.

    ,, ... 1935. *Ibid.*, lxi, 473.

WILSON, G. S. . . . . . . 1935. Medical Research Council Spec.
                                     Rep. Ser., no. 206, *London.*

THE EFFECT OF ASBESTOS ON TISSUE CULTURES: A COMPARATIVE STUDY WITH QUARTZ AND COAL DUST

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(Plates XX and XXI)

The effect of silica compounds has been widely studied in animal experiments, yet the manner in which they act upon living cells is still uncertain. Of the various silicious minerals, quartz is the principal pathogen, and most authorities believe it to act on the tissues by liberating silicic acid which causes necrosis. Gye and Kettle (1922), Gardner and Cummings (1933), and Fallon and Banting (1935) found that powdered quartz caused necrosis when injected subcutaneously. Accordingly the fibrosis which characterises silicosis came to be regarded as the cicatricial aftermath of this necrotising process.

Asbestosis also is characterised by fibrosis of the lungs, and the inference drawn from the parallel with silica—asbestos being a compound silicate of magnesium—is that powdered asbestos, like silica, induces necrosis as an initial effect; but whether it can be considered to owe its pathogenicity to its silica content is not certain (*cf.* King, Clegg and Rae, 1946).

Tissue culture provides a method of approach for the study of pneumokoniosis *in vitro*. Several authors (Lauche, 1931; Franks and Watt, 1934; Kasten, 1938-39) used coal and quartz dusts, but no work appears to have been done with asbestos in tissue culture experiments.

METHODS

From some preliminary experiments it appeared that the action of asbestos could best be ascertained by comparing its effect with that of coal and quartz. The latter, being more readily phagocytosed than asbestos and more easily distinguished in tissue cultures, were good indicators of the effect produced and served as a kind of control.

Technique of the tissue culture experiments

General technique

Culture media. Fowl plasma and extract of chick embryos of 11-12 days' gestation.

Tissues implanted. Lung or heart of 11-12 day-chick embryos. Lung or heart of 2-3 weeks old rat embryos. Human fœtal lung from cases of Cæsarian section. Leucocytes from fowl, rat or human blood prepared by Carrel's method.

Incubation period. (a) At 39° C. and subcultured 48-72 hourly. (b) Initial incubation at 39° C. for 38-72 hours, and then kept at room temperature for 2-4 weeks (longer for prolonged observation). Tissue cultures can be kept alive in this way over a long period of time. We have seen Brownian movement as long as 14 days, and "pulsation"—due probably to contracting vessels—as long as 17 days in culture.

Dusting technique

Dusts used. (1) Canadian asbestos 2·5 μ and 5 μ ; (2) quartz ; (3) steam coal "S4" ; (4) anthracite "A1" (ref. no. 94. U.S. 10). It is very difficult to cut asbestos and even dust of the smaller size contains a number of long fibres. The asbestos and control dusts were applied in dry powdered form or in sterile fluid suspension.

Alternative methods of dusting. (1) A cloud of dust produced in a specially devised apparatus was allowed to settle on the implant (Lauche's method). (2) The dust was applied on a coverslip by drying a drop of the dust suspension (Frank's method). (3) Injection of the suspension directly into embryonic lung tissue. (4) Intratracheal injection of dust suspensions into the embryo and subsequent division of the injected lung into several fragments which were then implanted.

In order to ensure, as far as possible, uniformity of reaction, a fragment of embryonic tissue was divided into four approximately equal parts and each part then dealt with separately ; one segment acted as undusted control, the others were dusted.

Methods 1 and 2 appeared to be especially suitable for the study of phago-cytosis but suffered the disadvantage that the applied dust lay in a different layer. In order to secure a more intimate contact between the cells and the dust, the injection technique was developed. In method 3, three of the four parts of the tissue fragment were each treated with 1 ml. of saline containing 10 mg. of dust : namely part 1 with asbestos, part 3 with quartz, and part 4 with anthracite or steam coal, while part 2 (control) received no dust.

The same suspensions were injected intratracheally in 3-week rat embryos. The trachea was laid open under strictly aseptic conditions, and 1·2 ml. of the dust suspension injected into the trachea and lungs. The dust appeared widely distributed in the alveoli and in the tissue. Soon wandering cells appeared attacking the particles. This method proved particularly suitable for the observation of the effect of asbestos on the motility of phagocytes. In 28 experiments about 500 slide cultures and 50 flask cultures were examined.

RESULTS

The following summary of observations may profitably be considered under several headings.

1. *Beginning of phagocytosis*

Steam coal. Phagocytosis was usually early. In many cultures it was already well marked after only 16-18 hours' incubation.

Anthracite. Phagocytosis, sometimes early, was generally delayed compared with steam coal. There was marked phagocytosis after about 48 hours' incubation.

EFFECT OF ASBESTOS ON TISSUE CULTURES

FIG 1 —Tissue culture of chicken embryo heart dusted with asbestos, showing growing edge   ×270

FIG 2 —Higher power view, showing detail   Well developed fibroblasts surrounding clusters of asbestos fibres   ×730
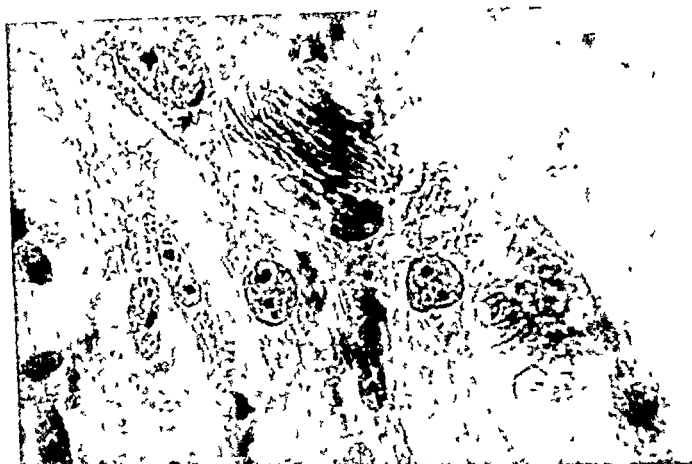
EFFECT OF ASBESTOS ON TISSUE CULTURES

FIG 3.—Advancing margin of fibroblasts from a tissue culture of 5 days growth
Cluster of asbestos fibres (above centre) within a cell the outline and nucleus
of which are clearly visible (? wandering cell or fibroblast) Other clusters of
asbestos fibres (2 5μ in length) to right are also intracellular Mitoses on left
and right of field ×875

FIG 4.—Tissue culture at 28 days Two
appearance a †   of characteristic
of fat ×   as many granules

Quartz. In most of the experiments quartz was readily engulfed by wandering cells, although this was sometimes delayed in comparison with steam coal. After 48 hours' incubation phagocytosis reached a high rate, almost equal to that of steam coal.

Asbestos. The wandering phagocytes were indifferent at first. Indeed, at no time did they show avidity for the particles and much asbestos remained untouched though within easy range of the wandering cells. After 48-72 hours there was fairly active phagocytosis of individual fibres of $2 \cdot 5 \mu$ length, but little attempt was made to cope with clusters or long fibres. None of the cells became heavily loaded with asbestos.

2. *Rate of phagocytosis*

The coal and quartz were easily engulfed and phagocytosis reached a high rate after 48 hours. We could not see any appreciable difference between quartz and coal at this stage. Asbestos, on the other hand, showed a very low rate of phagocytosis, and indeed in some experiments it was difficult to find cells containing asbestos fibres, though the controls showed crowds of phagocytes containing coal or quartz particles.

3. *Size of particles*

Even large clumps of coal and large crystals of quartz were readily engulfed, but only short asbestos fibres of $2 \cdot 5 \mu$ were seen to be taken up to any considerable extent. In one experiment only have we seen starlike clusters of asbestos fibres in the cells. In some cultures, especially of chick leucocytes and human embryo lung, a few long fibres became engulfed by large phagocytes; but we have quite commonly observed several large coal particles or quartz crystals in numerous phagocytes, no matter of what provenance. No appreciable change in the shape of the engulfed particles could be seen.

4. *Effect on the growth of the tissue*

When more heavily dusted, cultures did show restriction and sometimes suppression of growth, but this seemed to be a mechanical rather than a chemical effect. The fibroblasts showed a striking indifference to dusting with coal, quartz or asbestos. Neither the rate nor the extent of growth was appreciably different from that of the control cultures. Individual particles as well as clusters were either by-passed or enveloped, all without noticeable detriment to the cells (figs. 1-4). The development of lung alveoli likewise showed no impairment.

5. *Effect on phagocytes*

No vacuolation was seen. Asbestos-laden cells remained apparently unchanged over a long period of observation. Cells containing fat as

well as asbestos fibres were often observed (fig. 4). In comparison with coal phagocytes the fatty change appeared to be less advanced in asbestos-laden cells. Coal-laden cells soon showed marked fatty change and often broke down, releasing the engulfed coal. Since asbestos as well as quartz phagocytes appeared to remain intact for a longer period, this would seem to confirm the observation of Franks and Watt that silica might have a slowing-down effect on the metabolism of the cell. No necrosis was seen.

Besides the delayed beginning and slow rate of phagocytosis and the apparently slower fatty change in asbestos cultures, asbestos-laden cells showed decreased motility.

6. *Effect on the motility of wandering cells*

This could best be observed in the cultures in which the dust was applied through injection of a dust suspension, especially by intratracheal injection. As mentioned above, a very good dusting effect could be achieved with this technique, and intimate contact between dust and cell assured. As all the dust was originally situated within the implant, any dust appearing outside the tissue must have been carried outwards by the wandering cells.

Dust-laden cells could first be seen in the alveoli and in the tissue. Coal-laden cells, especially those containing steam coal, soon appeared outside the tissue and reached a considerable distance from the tissue implant. Cells containing quartz or asbestos remained stationary in the tissue. If they sometimes did migrate outwards they never reached the same distance.

Discussion

Tissue-culture experiments necessarily extend over a very brief period. It must be admitted that they bear little relation to what happens in naturally-occurring pulmonary asbestosis. The apparent lack of any toxic effect on living cells in tissue cultures conveys an impression of innocuousness which is contrary to the well-known dangerous nature of asbestos dust when inhaled in large amounts by man over a long period of time. Changes in the asbestos fibre in the lungs take place very slowly and the first apparent change is the commencing formation of the asbestos body. A core of the original asbestos fibre remains in the asbestos body even after many years. It was not to be expected, therefore, that there would be any observable alteration in the fibres of asbestos during the few weeks of the tissue culture experiments. Beger (1934 *a* and *b*) showed the asbestos body to be formed of coagulated protein on the surface of the asbestos fibre, the silica content of the asbestos having become dispersed into this protein sheath. Such a release of silica from the asbestos fibre would lead eventually to a reduction of its size and probably to some toxic effect

on surrounding tissues or an engulfing cell. Such effects have not been observed in the present work, and it must be concluded that tissue culture experiments have not led us to a better understanding of the mechanisms involved in the production of asbestosis in the human lung.

SUMMARY

1. Phagocytosis, even of small particles of asbestos, took place slowly in tissue cultures and a considerable portion of the asbestos dust, approximately 50 per cent., remained untouched, though within range of phagocytes throughout the experiments. Compared with coal or quartz, asbestos was less readily phagocytosed, particle size being equal.

2. Asbestos particles in small amount had little or no toxic effect on living cells in tissue cultures. The growth of fibroblasts was neither retarded nor accelerated and no necrosis of cells was observed as a result of contact with asbestos particles, while fatty change appeared to occur more slowly in asbestos phagocytes than in coal phagocytes.

3. Asbestos particles underwent no visible change, and in particular no visible diminution in size, after having been ingested by phagocytes for as long as 5 weeks.

4. The motility of wandering phagocytes was decreased after the ingestion of asbestos particles. This effect, not necessarily of a toxic character, may be deleterious in the case of cells within the lungs, since it tends to restrict the migration of cells and so favour the retention of particles within the lungs.

5. Particles under 5 μ in length restricted cellular motility more than larger particles, for the reason that they were more readily phagocytosed. It would seem that wandering phagocytes are rarely able to ingest particles larger than 10 μ. Giant cells sometimes formed in tissue cultures, apparently by fusion of two or more ordinary phagocytes, and were then able to engulf larger particles or clusters of particles.

6. There was no evidence of the formation of asbestos bodies in these experiments, nor have we noted the appearance of brown granular pigment like that seen in the lungs of experimental animals.

REFERENCES

BEGER, P. J. . . . . . . 1934a. *Dtsch. med. Wschr.*, lx, 231.

„ . . . . . . 1934b. *Med. Klin.*, xxx, 1222 and 1258.

FALLON, J. T., AND BANTING, F. G. 1935. *Canad. Med. Assoc. J.*, xxxiii, 404.

FRANKS, W. R., AND WATT, A. J. 1934. *Trans. Roy. Soc. Canad.*, v, 2843.

GARDNER, L. U., AND CUMMINGS, 1933. *Amer. J. Path.*, ix, 751.
D. E.

GYE, W. E., AND KETTLE, E. H. . 1922. *Brit. J. Exp. Path.*, iii, 241.

KASTEN, W. . . . . . . 1938-39. *Arch. Gewerbepath. Gewerbehyg.*, ix, 337.

KING, E. J., CLEGG, J. W., AND 1946. *Thorax*, i, 188.
RAE, V. M.

LAUCHE, A. . . . . 1931. *Verhandl. dtsch. path. Gesellsch.*, xxvi, 107.

THE IMMUNOLOGICAL ASPECTS OF EXPERIMENTAL *HÆMOPHILUS PERTUSSIS* INFECTION

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THE problem of immunity to *Hæmophilus pertussis* infection is obscure and in spite of considerable published work there is little concrete evidence and much difference of opinion on the following essential points : (*a*) whether the apparent immunity in man, either that produced by an attack or that usually present in adults with no history of an attack, is a specific immunity ; (*b*) whether this immunity, if any, is antibacterial, antitoxic, or a combination of both ; and (*c*) what are satisfactory laboratory tests for the antigenicity of *H. pertussis* vaccines and the antibacterial or antitoxic values of immune sera. The purpose of this investigation was to evaluate the merits of the various laboratory methods used, to determine the nature of *H. pertussis* infection in mice, and to see what conclusions might be drawn about the probable efficacy of active and passive immunisation in whooping cough.

LITERATURE

The clinical value of active immunisation with vaccine has recently been reviewed for the American Council of Pharmacy and Chemistry by Felton and Willard (1944), who concluded that a significant protection was conferred as measured by a reduction in the attack rate and severity of the disease. This finding is by no means universally accepted and recent trials such as those by McFarlan, Topley and Fisher (1945) have shown little difference between vaccinated and control groups. Prophylactic and therapeutic serum treatment has also been reviewed by Felton (1945), who concluded that serum, particularly human hyperimmune serum, was effective in prophylaxis and treatment. Sera do not appear to have been used extensively in this country.

In the laboratory the mouse-survival test involving the intra-peritoneal injection of living organisms has been extensively used for the demonstration and comparison of the antigenic activity of *pertussis* vaccines and the protective value of immune sera. Cruickshank and Freeman (1937), Silverthorne (1939), Eldering (1942) and others have used this technique for vaccines, but an examination

of the protocols, wherever reasonable groups of mice have been injected, shows that the order of protection is only about 50 per cent. survival against an infecting dose which just kills all or the majority of the control groups.

The antibacterial value of immune serum has also been determined by this method, and Holm and Bunney (1942, p. 35) have described a provisional unit as that amount of serum which will protect *50 per cent. of mice from death* when a dose of 2 M.L.D. of culture is injected intraperitoneally. Groups of 3 mice and two or three dilutions of serum are used for the test, and no evidence is produced that serum will protect against a larger infecting dose.

The intranasal infection of mice following intraperitoneal or subcutaneous injection of vaccines has also been used to demonstrate active immunity (North, Anderson and Graydon, 1941). Although some protection has been demonstrated, it is of a low order and the results are further complicated by the difficulty of giving a uniform infecting dose. Antibacterial serum has been shown to protect against a small infecting dose, and serum containing antitoxin and no antibacterial antibody is also capable of protection under certain conditions (Evans, 1944). Dow (1940) has demonstrated that active immunisation by the intranasal route is more effective than by the intraperitoneal route against intranasal infection. However, North and Anderson (1942) have shown that the increased effectiveness is non-specific and associated with histological changes in the lung. Prophylactic immunisation of human subjects against whooping cough by the intranasal route is probably impractical but these results suggest an *interesting speculation* on the possible insusceptibility of adults to the disease.

METHODS

Preparation of H. pertussis *suspensions*

Phase I *H. pertussis* strains were maintained as dried cultures and recovered as required. The criteria used for phase I were : (*a*) virulence such that 1000-2000 million organisms killed at least five of a group of ten 20-g. mice injected intraperitoneally ; (*b*) specific agglutination to titre with phase I serum ; and (*c*) inability to grow on nutrient agar. Cultivation was on Bordet-Gengou medium containing 33 per cent. horse blood for 48 hours in Roux bottles, the growth being scraped off and suspended in 0·9 per cent. saline. The suspension was killed by heat or preservative and diluted to the required opacity.

Preparation of antibacterial serum

Rabbits were injected intravenously at 3- or 4-day intervals with 4 doses of 0·4, 0·8, 1·0, and 1·0 ml. per kilo. body weight of a suspension in 0·5 per cent. phenol-saline containing 10,000 million organisms per ml. They were bled 7 days after the last injection and the sera preserved with 0·25 per cent. phenol. This procedure always gave sera of high agglutination titres.

Preparation of toxin

Toxin was prepared from frozen and thawed, dried and ground bacilli by the method described by Evans and Maitland (1937). *The final product was*

suspended in 0·9 per cent. saline, 5 mg./ml., and centrifuged ; 0·1 ml. of supernatant was lethal for a 20 g. mouse on intraperitoneal injection, and the M.R.D. on intracutaneous injection in a rabbit was of the order of 0·006 ml.

Preparation of antitoxic serum in rabbits

Pertussis endotoxin is a poor antigen and a large number of subcutaneous injections were required to produce detectable quantities of antitoxin in rabbits. Fourteen injections were given subcutaneously, beginning with 1·0 ml. of a 1 : 5 dilution and increasing to 1·0 ml. of undiluted toxin. Some rabbits developed ulcers, but all remained in good health. The serum neutralised toxin according to the law of multiple proportions, and the potency was such that 0·05 ml. of serum protected against 2 M.L.D. of toxin injected intraperitoneally into mice, and 0·005 ml. against 10 M.R.D. on intracutaneous injection into rabbits. The agglutination titre against phase I organisms was low, usually about 1 : 40. When required, the agglutinins were absorbed by adding an equal volume of a suspension of 20,000 million killed organisms per ml., heating for 2 hours at 55° C. in a water-bath, and leaving overnight in the cold room, the organisms being finally removed by centrifuging. The resulting serum contained no agglutinins but retained its antitoxic properties.

Intranasal infection of mice

Mice were infected intranasally by the method of Burnet and Timmins (1937). They were lightly anæsthetised with a mixture of one part chloroform and two parts ether, and 0·05 ml. doses were given intranasally with a 1-ml. pipette graduated to 0·01 ml. For this purpose it was found convenient to have a 1-ml. syringe attached to the pipette, both being held in position with a retort stand and clamps.

Determination of the number of viable organisms in the blood of infected mice

(1) *By bleeding from the tail.* The tail was washed with spirit, excess being removed with sterile cotton wool, passed rapidly through a bunsen flame and immediately rubbed with sterile cotton wool. The extreme tip of the tail was snipped off with sterile scissors and a drop of blood, which usually appeared immediately or after a little massage, was removed with a small sterile loop. The blood was plated on Bordet-Gengou medium containing 1 unit per ml. of penicillin to inhibit growth of Gram-positive contaminants. The plates were incubated for six days and the number of colonies counted.

(2) *By bleeding from the axillary blood vessels.* A rapid and convenient method has been found of obtaining a measured volume of blood from mice. The animals were killed by coal gas and pinned on a post-mortem board, and the hair was moistened with hot water. An incision was made with sterile scissors along the median line of the thorax ; the left flap of skin was held back with forceps and with another pair of sterile scissors the subcutaneous layer of tissue was dissected and the main axillary vessels cut. The blood which collected in the axillary space was removed with a sterile calibrated capillary pipette ; up to 1 ml. amounts of sterile blood may regularly be obtained in this way. The method is very suitable for the routine passage of strains in mice and is much quicker than the usual routine of obtaining blood from the heart. In the present investigation usually 0·01 ml. of blood was plated on Bordet-Gengou medium.

Determination of the number of viable organisms in the lungs of infected mice

Mice infected intranasally with *H. pertussis* were killed by coal gas, and the whole of one lung was removed from each animal with aseptic precautions and placed in a one-ounce screw-capped bottle one-third filled with glass beads and containing 10 ml. of broth. The bottles were shaken for a few minutes and measured volumes, usually 0·01 ml., of broth were plated on Bordet-Gengou medium containing 1 unit per ml. of penicillin. The plates were incubated for six days and the number of colonies counted.

Agglutination test

0·5 ml. of serum dilution and 0·5 ml. of antigen were incubated in a water-bath at 55° C. for 2 hours, one-third of the fluid of the tubes being immersed.

EXPERIMENTAL

The nature of the intraperitoneal infection of mice

The intraperitoneal method has been used extensively in some laboratories as a means of measuring antibacterial immunity, both active and passive, and the potency of anti-*pertussis* sera has been expressed in terms of provisional antibacterial units. But since the test has not been satisfactory in my hands, an attempt has been made to find out what, in fact, it measures.

In the first place there is a considerable range of survival after intraperitoneal injection of increasing numbers of living virulent phase I organisms into groups of mice. It is not possible to calculate the LD 50 with any sort of accuracy unless very large numbers of mice are used (this is clearly indicated in the survival rate of the control mice shown in the protocols). The use of mucin, always to be deprecated unless unavoidable, reduces the LD 50 only about 2 to 3 times.

Second, in view of the large numbers of organisms required to kill mice, it seemed likely that death was the result of toxæmia and not septicæmia. By counting the number of bacteria in the blood of mice after intraperitoneal infection, it was found that *H. pertussis* appeared in the blood stream a few minutes after injection and that the numbers quickly diminished (table I). There was no evidence of multiplication at any stage.

Passive immunity

The effect of antitoxic and antibacterial serum was investigated and it was found that whereas antibacterial sera gave only slight protection, at the most against one or two times the LD 50 dose, antitoxic sera gave good protection against large doses of organisms. The results of the passive protection tests are shown in table II.

It seems clear from these results that mice injected intraperitoneally with *H. pertussis* die of toxæmia which can be specifically neutralised

by antitoxin. Since antibacterial serum has little effect, this method of infection is of little value as a measure of antibacterial immunity.

TABLE I

H. pertussis *in the blood of mice infected intraperitoneally*

No of organisms injected intraperitoneally	No of colonies from one standard loopful of circulating blood after :—			
	$\frac{1}{4}$ hr.	5 hr.	24 hr.	48 hr.
4000 million	1297	202	131	Mouse dead
1000 ,,	315	125	28	,, 0 ,,
250 ,,	116	75	5	0
62 ,,	136	2	0	0
16 6 ,,	22	1	0	0

No of colonies from mouse infected with 1500 million organisms after :—							
5 mm.	15 mm.	$\frac{1}{4}$ hr.	1 hr.	2 hr.	3 hr.	4 hr.	5 hr.
143	455	775	121	119	65	30	30

TABLE II

Passive protection of mice against intraperitoneal infection

Serum (0 5 ml) given intraperitoneally 24 hr. before living culture	No of living organisms injected intraperitoneally (millions)				
	13,500	4500	1500	500	166
Antitoxic serum—rabbit (agglutinin titre 1 : 40 ; 1 ml. neutralises 512 M.R.D. toxin : rabbit intracutaneous test)	10	10	10	10	10
Antibacterial serum—rabbit (agglutinin titre 1 : 2508 ; 1 ml neutralises <1 M.R.D. toxin : rabbit intracutaneous test)	*0*	*4*	*7*	*10*	*10*
Controls (no serum)	0	0	5	8	10

The figures show numbers of mice surviving out of groups of 10.

Active immunity

In a large number of tests it was found impossible to compare the antigenic values of vaccines by the intraperitoneal survival method. The degree of protection was slight and the results were irregular and not reproducible ; but by counting the number of organisms appearing in the blood at various times after infection, it is possible to demonstrate the presence of some antibacterial bodies and to demonstrate major qualitative differences between vaccines.

Table III shows the effect of active immunisation with various doses of phenolised vaccine on the number of organisms appearing in

the blood. Vaccination prevents the majority of the organisms from appearing in the blood, and those which appear are killed more rapidly in vaccinated than in non-vaccinated control mice.

TABLE III

Active immunity in mice against intraperitoneal infection

Vaccine, 100 million phenol-killed organisms injected intraperitoneally at weekly intervals; living culture 7 days later		Symbols represent degrees of transient bacteriæmia of individual mice at intervals after intraperitoneal injection of 1000 million organisms	
		Vaccinated groups	Control groups
1 dose vaccine	½ hr.	++ ++ + + + ± ± 0 0 0	++ ++ ++ ++ ++ ++ ++ ++ + ± ± 0
	5 hr.	+ ± ± ± 12 0 0 0 0 0	++ ++ ++ + ± ± ± ± ± 0
	24 hr.	D + ± 2 0 0 0 0 0 0	D D D D D + ± ± ± ±
2 doses vaccine	½ hr.	++ ++ ++ ++ + + + ± ± 3	++ ++ ++ ++ ++ ++ ++ ++ ++ ++
	5 hr.	± ± ± ± 3 1 0 0 0 0	++ ++ ++ ++ + + + ± ± ±
	24 hr.	2 0 0 0 0 0 0 0 0 0	± ± 8 8 6 3 3 3 2 1
4 doses vaccine	½ hr.	+ + + + + ± 0 0 0 0	++ ++ ++ ++ ++ ++ + + + ±
	5 hr.	1 0 0 0 0 0 0 0 0 0	++ ++ + + + ± ± ± ± ±
	24 hr.	0 0 0 0 0 0 0 0 0 0	D D + ± 4 4 3 2 0 0
6 doses vaccine	½ hr.	20 20 0 0 0 0 0 0 0 0	++ ++ ++ ++ ++ ++ ++ ++ ++ 0
	5 hr.	D 0 0 0 0 0 0 0 0 0	++ + + + + + 2 2 0 0
	24 hr.	D 0 0 0 0 0 0 0 0 0	D D + + + 5 5 0 0 0

++ = >100 colonies from standard loopful of tail blood
+ = 100-50 „ „ „ „ „ „ „
± = 50-25 „ „ „ „ „ „ „
Numerals = colony counts (under 25)
D = mouse dead

With 6 doses of 100 million phenolised organisms given at 4-day intervals and the mice tested 7 days later probably very few organisms ever appear in the blood. The absence of *H. pertussis* from the circulation appears to have little effect on the survival rate. With groups of ten mice immunised with 6 doses of 100 million killed organisms as in the previous experiment, the number of mice surviving intraperitoneal injection of 13·5, 4·5, 1·5 and 0·5 thousand million living organisms was 0, 8, 6, and 10 for the immunised, and 0, 3, 5 and 10 for the control groups respectively.

Considerable emphasis has been laid by some workers on the effect of preservatives, heat, and so forth on the efficacy of phase I *H. pertussis* vaccines. Using the tail-count method, the results indicate that there is no major difference in antigenicity between heat-treated, formolised, phenolised, merthiolated or alum-precipitated phase I vaccines, but phase IV vaccines are not effective. Table IV indicates the type of results obtained.

There is slight evidence that repeated washing with saline diminishes the antigenic properties of vaccines, the tail counts with unwashed vaccine being persistently lower than with vaccine washed six times with saline. There was no suggestion that even six washes had

removed the greater part of the antigenicity as measured by this test.

TABLE IV

Protection given by various types of H. pertussis *vaccine against intraperitoneal infection*

(Mice received 1 injection subcutaneously of 1000 million organisms; living culture two weeks later)

Vaccine	No. of organisms in infecting dose (millions)				* No. of colonies from tail blood (average of ten mice)
	13,500	4500	1500	500	
Heat-killed phase I	0	0	9	10	10
Phenol-killed phase I . . .	0	1	5	9	9
Phenol-killed alum-precipitated phase I	1	2	6	10	8
Phenol-killed phase IV . . .	0	1	7	10	120
Controls (no vaccine) . . .	1	0	4	10	190

The figures in the middle section show the numbers of mice surviving out of groups of 10.

* Blood taken one hour after intraperitoneal injection of 1000 million living organisms.

Intranasal infection of mice

The picture is entirely different if mice are infected intranasally with virulent *H. pertussis*. The organisms multiply in the lungs and remain viable there for a considerable period. Table V, which gives

TABLE V

Survival of H. pertussis *in the lungs of mice infected by the intranasal route*

Mice killed after	Infecting dose of 0·05 ml. of	
	200 million organisms/ml.	1 million organisms/ml.
1 hour	300	10
1 day	400	50
2 days	1000	40
3 ,,	2000	40
7 ,,	10,000	5000
14 ,,	300,000	10,000
21 ,,	10,000	1000
28 ,,	2000	100
35 ,,	100	2
42 ,,	100	2
49 ,,	10	2
56 ,,	2	0

The numerals show the average number of colonies on plates for groups of 5 mice.

the results of one experiment, shows that the organisms multiply in the lungs, reach a maximum in 7-14 days and slowly die out; they may be recovered up to 56 days after infection.

Table VI shows the protection against intranasal infection with rabbit antibacterial serum and rabbit absorbed antitoxic serum, 0·5 ml. of serum having been given intraperitoneally 24 hours before infection by the intranasal route with 0·05 ml. of a broth suspension of living organisms.

TABLE VI

Passive protection of mice against intranasal infection

Serum (0·5 c.c.) given intra-peritoneally 24 hours before living culture	+Infecting dose (intranasal) of 0·05 ml. of			
	1000 million/ml.	200 million/ml.	40 million/ml.	1·6 million/ml.
Rabbit (antibacterial) .	5 (1)	5 (2)	5 (1)	5 (0)
Rabbit (absorbed antitoxic)	4 (4)	3 (3)	4 (1)	5 (0)
Controls (no serum) . .	3 (3)	3 (3)	3 (3)	5 (4)

Figures show the numbers of mice surviving 14 days out of groups of 5.

In brackets : no. of survivors with *H. pertussis* isolated from lung when killed on 14th day.

These results indicate that antibacterial immunity plays some part in the protection of mice against intranasal infection ; this is in agreement with published work. While antitoxin also may have some effect in preventing the multiplication of organisms in the lung, antibacterial immunity appears to be rather more important than antitoxic immunity where the intranasal infection of mice is concerned.

Active immunisation of mice against intranasal infection

Groups of mice injected subcutaneously with 0·5 ml. of a suspension of 2000 million phenol-killed phase I organisms per ml. were infected

TABLE VII

Active immunisation of mice against intranasal infection

(1000 million phenol-killed organisms injected subcutaneously ; living culture intranasally seven days later)

Infecting dose 0·05 ml. intranasally of a suspension containing	Mice surviving out of groups of 20 mice		No. of organisms in lungs of infected mice after			
			1 day		7 days	
	Immunised	Control	Immunised	Control	Immunised	Control
20,000 million organisms/ml. .	1	0	...	...	...	...
2000 ,, ,, ,, .	6	0	*800	5000	∞	∞
200 ,, ,, ,, .	9	1	200	400	500	∞
20 ,, ,, ,, .	10	3	50	300	1000	4000

∞ = >250,000

* Average number of colonies, estimated from plate counts, from groups of 10 mice. colonies.

intranasally with different amounts of living culture seven days later, and survival rates determined.

The number of organisms in the lungs of actively immunised mice was also determined ; the results are shown in table VII.

These results indicate a degree of protection which is not, however, sufficient for survival after a large infecting dose.

There is also a considerable reduction in the number of organisms in the vaccinated mice, particularly with a small infecting dose. Quantitative results and comparisons of vaccines are difficult by this method since it is impossible to get a uniform infecting dose.

HORSE IMMUNE SERUM (ANTIBACTERIAL AND ANTITOXIC)

From the results reported above it would seem that passive immunisation with serum and active immunisation with vaccine can slightly modify experimental infection in mice. The protection is of a low order, and by comparison with the laboratory evidence for such vaccines as *Bacterium typhosum* and *Pasteurella pestis*, which have proved effective in practice, the results are not very encouraging. Since active immunisation in man has not given clear-cut results, it was thought that additional evidence could be obtained by passive immunisation with a hyperimmune serum, for this would give some idea of the importance of specific immunity in whooping cough. Consequently attempts were made to prepare in horses a serum of high antibacterial and antitoxic content.

Immunisation of horses

The antigen used for horses was *pertussis* toxin prepared by the method of Evans and Maitland, except that a suspension of bacterial bodies and toxin was used ; the excess of bacterial bodies was not removed by centrifugation. Three horses were given a three months' course of three injections a week ; the first few injections were formolised toxin, and the remainder toxin. The dose of antigen was gradually increased from 1 to 35 ml. After three months three 8-litre bleedings were taken from each horse. The horses were rested and then had a rapid course of alum-precipitated toxin consisting of five doses of 25, 50, 50, 75 and 75 ml. of antigen in two weeks. One week later a further series of three 8-litre bleedings was taken from each horse.

Concentration of horse antiserum. Since unconcentrated horse serum is unsatisfactory for human use because of severe serum reactions, the serum was concentrated by ammonium sulphate precipitation as described by Harms (1946). Limited clinical trials have shown that the sulphate-concentrated serum does not show any high degree of serum reactions. The protein content of the unconcentrated serum was about 7·0 per cent. and of the concentrated serum 15 per cent., with an increased activity of about 8 times as judged by agglutination and protection antibodies. There was a fourfold increase in purity.

PROPERTIES OF HORSE ANTISERUM (UNCONCENTRATED)

The properties of the horse antiserum were studied experimentally with unconcentrated serum in mice. There was little difference between the sera of the three horses used.

Agglutinin titre

The titre against phase I organisms was about 1 : 16,000. The sera showed a strong pre-zoning effect and there was no agglutination up to 1 : 100.

Antitoxin content (intracutaneous test in rabbits)

The antitoxin content of the horse serum was determined by the skin reaction in rabbits, and it was found that 1 ml. of serum neutralised approximately 10,000 M.R.D. of toxin. As previously shown in this paper, protection against living culture given intraperitoneally is due mainly if not entirely to this anti-endotoxin.

Effect on the number of organisms in the blood of mice of the intraperitoneal injection of living culture

0·25 ml. of serum diluted 1 : 10 in saline was injected intraperitoneally into a group of mice 24 hours before the intraperitoneal injection of 1000 million living organisms. The number of organisms present two hours later in a small loopful of blood was compared with the figure for a control group of mice receiving no serum. In a group of 10 treated mice the average number of organisms was 4 compared with 300 in the control group.

Protection of mice against living culture injected intraperitoneally

0·5 ml. of serum dilution was injected intraperitoneally 24 hours before the intraperitoneal injection of living culture. The potency of this serum was such that 0·05 ml. of serum diluted 1 : 100 protected about 50 per cent. of mice against a test dose of approximately 10 M.L.D. This protection was due entirely to the antitoxic content of the serum since a potent rabbit antibacterial serum showed no protection against this test dose.

Effect on the number of organisms in the lungs of mice after intranasal infection with living culture

Mice were injected intraperitoneally with 0·25 ml. of serum 24 hours before intranasal infection with 0·05 ml. of a suspension of living H. pertussis containing 200 million organisms per ml. Control and treated mice were killed one week later and the number of organisms in the lungs was determined. A comparison was made between rabbit antibacterial sera, phase I and phase IV, pooled adult human serum, and the horse immune serum containing antibacterial and antitoxic antibodies (table VIII).

It can be seen that all these sera reduce the average number of organisms in the lungs of mice but that the horse serum is by far the most effective.

An attempt was made to see if the horse serum was effective therapeutically. One group of 200 mice was injected intranasally with 0·05 ml. of a suspension of *H. pertussis* containing 100 million organisms per ml. At intervals of 1, 7 and 14 days after injection

TABLE VIII

Passive protection of mice against intranasal infection

(Comparison of antibacterial values of sera by counting organisms in lung one week after intranasal infection with 0·05 ml. of broth suspension (200 million organisms/ml.): 0·25 ml. serum given intraperitoneally 24 hours before culture)

Serum	Mouse no.									
	1	2	3	4	5	6	7	8	9	10
Rabbit antibacterial, phase I (agglutinin titre 1 : 10,000)	0	0	0	0	1000	7000	4000	4000	1000	500
Rabbit antibacterial, phase IV									10,000	∞
Adult human									1000	9000
Horse immune (agglutinin titre 1 : 20,000; 1 ml. neutralises 10,000 M.R.D. toxin in rabbit intracutaneous test)									0	0
Controls	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞

Numbers = estimated number of colonies from plate counts
∞ = >250 thousand

groups of 10 mice were given 0·25 ml. of horse serum intraperitoneally. Seven days after serum the treated mice and ten surviving control mice were killed and the number of organisms in the lungs determined. At the end of 14 days there were not sufficient mice to continue the tests, since a number of mice had died of *H. pertussis* infection. The protective value of the serum was shown by the fact that no serum-treated mice died. It is evident from table IX that the horse serum is effective in removing organisms from the lung after infection has been well established.

DISCUSSION

The difference in the survival rate between immunised and control mice after intraperitoneal injection of living cultures of *H. pertussis* has been extensively used for the demonstration of antibacterial immunity. The experiments reported in this paper have clearly shown that death following intraperitoneal injection of culture is due to a toxaemia which may be neutralised by specific antitoxin and that there is no evidence of multiplication of organisms in the mouse. Attempts have been made to increase the virulence of the organisms by injections of mucin (Mishulow *et al.*, 1939) and of starch (Powell and Jamieson, 1937); however, the average lethal dose has not been

appreciably altered by this technique, and the general conclusion as
to the mechanism of death from intraperitoneal infection need not
be modified.

TABLE IX

*Therapeutic effect of horse immune serum on mice infected
by the intranasal route*

(Mice infected intranasally with 0·05 ml. suspension of living *H. pertussis*
containing 100 million organisms/ml. 0·25 ml. horse immune serum
injected intraperitoneally at intervals. Seven days later, serum-treated
mice and control mice killed and the number of organisms in the lungs
determined)

	No. of colonies on plates from lungs									
	Mouse no.									
	1	2	3	4	5	6	7	8	9	10
Horse immune serum injected 24 hr. after culture	0	0	0	0	60	100	100	20	20	100
Control mice	∝	∝	∝	∝	∝	∝	∝	6000	6000	4000
Immune serum 7 days after culture	0	0	0	0	0	0	0	0	0	0
Control mice	∝	∝	∝	0	1800	1800	3000	4000	600	C
Immune serum 14 days after culture	0	0	0	0	0	0	0	0	100	200
Control mice	4000	4000	4000	2000	2000	2000	2000	6000	100	100

Numbers = estimated number of colonies from plate counts.
C = grossly contaminated.
∝ = >250 thousand.

The intraperitoneal survival test cannot· therefore be used as a
measure of antibacterial immunity. It is true that some protection
can be demonstrated if very large groups of mice are used, but it is
against only 1 or 2 minimal lethal doses and the results are very
irregular. The immunity immediately breaks down if the infecting
dose is increased. It is difficult to explain even this small degree of
protection ; possibly it is due to agglutination of organisms and
localisation of toxic substances in parts of the body where their effect
is not so lethal. The part played by toxin in human infection is
obscure, and so far no antitoxin has been demonstrated in the sera
of convalescents or immune persons ; in this respect the work of
Strean (1940) does not seem to have been confirmed. It is not possible
to draw any conclusions from the intraperitoneal survival method
except possibly that any antibacterial immunity, active or passive,
which can be demonstrated by this method is of an extremely low
order.

After intraperitoneal injection the organisms appear in the blood
stream in a very short time and then progressively diminish in
number. Both active and passive immunisation accelerate the
disappearance of organisms, and in some cases it appears that they

never reach the blood stream in appreciable numbers. Perhaps some tentative general conclusions may be drawn from this technique but it is not suitable for quantitative comparisons. Table III shows that active immunisation, particularly with a number of immunising doses, significantly reduces the number of organisms appearing in the blood of mice. A number of vaccines are compared in table IV, and in this connection it may be recalled that various authors have claimed differences in the antigenicity of vaccines associated with minor differences in technique, such as the species of blood used in the medium, the type of preservative and so on. It would seem that, qualitatively, heated-killed, phenolised, and alum-precipitated vaccines have the same order of antigenicity, but that results with phase IV vaccines are not significantly different from those in the control group.

An entirely different type of infection is obtained with intranasal administration under light anæsthesia. The organisms multiply in the lungs and reach a maximum number in from 7 to 14 days; thereafter they steadily diminish but may be recovered from the lung up to 56 days after infection. Histological examination shows a pneumonic type of infection; staining with Twort's stain shows large numbers of organisms not demonstrable in Gram-stained preparations. A good description of the histological picture has been given recently by Hoyle and Orr (1945).

Active immunisation of mice will reduce the death rate from intranasal infection and will accelerate the disappearance of organisms from the lungs. It is not effective against many lethal doses, but is effective against a large number of minimal lung-infecting doses, that is, the smallest inocula which will allow organisms to be recovered from the lung after several days. The results of the method are irregular and do not allow accurate comparisons of antigenicity; this is not surprising in view of the difficulty of obtaining a uniform infecting dose, since mice inhale and exhale the inoculum to varying degrees. It is difficult to apply the results to human infection with *H. pertussis*; but it would seem that the protection would be of a low order, since the mice are not protected against a number of lethal doses. These experiments show that in the mouse it is possible by vaccination to increase very considerably the ability of the animal to eliminate rapidly a small infecting dose. On the other hand, the vaccinated animal is still incapable of dealing with a large infecting dose.

This may partly explain the divergent clinical results obtained with vaccination in a closed community, as in the British trials (McFarlan *et al.*, 1945): the subjects might well have been exposed to a large infecting dose.

Among the general population, under ordinary home conditions, the infecting dose must often be much smaller and the vaccinated child might well escape an attack.

Antibacterial sera are effective in reducing the mortality against a lethal infecting dose given intranasally, and in quickly eliminating the organisms from the lungs of mice given a sub-lethal dose. Antitoxin also has an appreciable effect, in these experiments rather more than in those of Evans. Here again the protection is against only a few lethal doses, but it is effective in rapidly eliminating organisms from the lungs when the infecting dose is many times the minimal infecting dose. A hyperimmune horse serum containing both antibacterial and antitoxic substances gives quite good protection and is effective not only prophylactically but also therapeutically, even when the lung infection has reached an advanced stage.

Taken as a whole, these results do not seem very encouraging for the success of specific prophylaxis or serum treatment in whooping cough. There is not the clear-cut protection against a large number of infecting doses in mice which has been obtained with the few organisms against which specific prophylaxis is successful—*Bact. typhosum* and *P. pestis*, for example. It may be that specific immunity plays a part in whooping cough, since hyperimmune serum has such a pronounced effect in animals. With such a serum, passive immunisation of groups of child contacts might afford tangible results, though the difficulties of such a trial are very great; if it could be clearly shown that passive immunisation is sufficient to protect against whooping cough, this would stimulate further research on active immunisation.

Hyperimmune sera may be of value in treatment but as originally observed by Bordet and Gengou (1909) the severe stage of whooping cough may continue long after *H. pertussis* can be isolated.

The part played by toxin in *H. pertussis* infection is not very clear; one factor which makes investigation difficult is the poor antigenicity of the toxin as shown by laboratory tests and by the absence of circulating antitoxin in the sera of convalescents. There is some suggestive evidence that toxin may play a part in infection. Thus, Evans has shown that mixtures of antitoxin and organisms injected intranasally are less infective than organisms alone, and suggests that the antitoxin may alter the invasiveness of the organism.

In a few experiments with the tail-bleeding technique, antitoxic serum from which antibacterial substances had been absorbed had much the same effect as antibacterial serum in preventing the organisms from appearing in the blood of mice infected intraperitoneally. This may suggest that *H. pertussis* invades by virtue of its toxin. Further, the prophylactic injection of antitoxin alters the character of the lesion in the rabbit and guinea-pig following intracutaneous injection of living cultures; the reactive lesion due to the toxin does not appear. Also, horse serum containing antibacterial and antitoxic immune bodies seems more effective than either antitoxic or antibacterial serum separately in modifying the infection following intranasal injection of living culture.

SUMMARY

Mice infected intraperitoneally with *H. pertussis* die of a toxæmia which may be specifically neutralised by antitoxin. There is no evidence of multiplication of organisms in the blood at any stage. Consequently the intraperitoneal method cannot be used as a measure of antibacterial immunity.

Active immunisation with vaccine will prevent *H. pertussis* from appearing in the blood of mice infected intraperitoneally, although it has little effect on the survival rate. With this technique it is possible to demonstrate major qualitative differences between antigens. Heat-, phenol-, formalin- and merthiolate-killed, and alum-precipitated phase I vaccines had the same order of antigenicity, whereas phase IV vaccines were non-antigenic.

With intranasal infection of mice the organisms multiply in the lungs and a toxic pneumonic type of infection occurs. Active and passive immunisation will protect against a number of minimal infecting doses and against a few lethal doses. Some protection was demonstrated with antitoxin, but antibacterial antibodies were more important. The best results were obtained with a hyperimmune horse serum containing both antibacterial and antitoxic antibodies. This serum was effective prophylactically and therapeutically even when infection was well advanced.

The part played by toxin in *H. pertussis* infection is obscure but there is some evidence that the organisms invade the tissues by virtue of their toxin.

The mouse experiments do not indicate that active immunisation against whooping cough is likely to be very effective. There is not the clear-cut protection against a large number of fatal doses which has been demonstrated in the laboratory animal for the few bacterial diseases against which active immunisation is effective.

REFERENCES

BORDET, J., AND GENGOU, O.   . 1909. *Ann. Inst. Past.*, xxiii, 415.
BURNET, F. M., AND TIMMINS, C. . 1937. *Brit. J. Exp. Path.*, xviii, 83.
CRUICKSHANK, J. C., AND FREE- 1937. *Lancet*, ii, 567.
MAN, G. G.
DOW, R. P. . . . . 1940. *Canad. Publ. Hlth. J.*, xxxi, 370.
ELDERING, G. . . . . . . 1942. *J. Bact.*, xliii , 78.
EVANS, D. G. . . . . . . 1944. This *Journal*, lvi, 49.
EVANS, D. G., AND MAITLAND, 1937. *Ibid.*, xlv, 715.
H. B.
FELTON, H. M. . . . . . . 1945. *J. Amer. Med. Assoc.*, cxxviii, 26.
FELTON, H. M., AND WILLARD, 1944. *Ibid.*, cxxvi, 294.
C. Y.
HARMS, A. J. . . . . . . 1946. *Nature*, clvii, 514.

HOLM, A., AND BUNNEY, W. E. .   1942.   *J. Immunol.*, xliv, 33.

HOYLE, L., AND ORR, J. W. . .   1945.   This *Journal*, lvii, 441.

MCFARLAN, A. M., TOPLEY, E.,   1945.   *Brit. Med. J.*, ii, 205.
     AND FISHER, M.

MISHULOW, L., KLEIN, I. F., LISS,   1939.   *J. Immunol.*, xxxvii, 17.
     M. M., AND LEIFER, L.

NORTH, E. A., AND ANDERSON, G.   1942.   *Med. J. Austral.*, ii, 228.

NORTH, E. A., ANDERSON, G., AND   1941.   *Ibid.*, ii, 589.
     GRAYDON, J. J.

POWELL, H. M., AND JAMIESON,   1937.   *J. Immunol.*, xxxii, 153.
     W. A.

SILVERTHORNE, N. . . . . . .   1939.   *Canad. Med. Assoc. J.*, xli, 263.

STREAN, L. P. . . . .   1940.   *Ibid.*, xlii, 525.

A CASE OF IDIOPATHIC METHÆMOGLOBINÆMIA TREATED BY ASCORBIC ACID AND METHYLENE BLUE

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METHÆMOGLOBINÆMIA may be produced in normal persons by the action of certain chemical poisons and bacteriological toxins, and many such cases have been described in the literature. Removal of the toxin results in a gradual return of the blood pigment to normal.

Records also exist of a small number of cases of methæmoglobinæmia occurring as an idiopathic condition, usually present from early years. From the instances in which a family history has been obtained, this would appear to be inherited as a Mendelian recessive character. Bensley *et al.* (1938), who described the condition in a French-Canadian brother and sister, found records of five previous cases in the literature. Lian *et al.* (1939) reported the abnormality in two brothers, and Deeny *et al.* (1943) described the first case to be studied in the British Isles.

The condition is characterised by marked cyanosis of a slightly brownish tint, due to the presence of a considerable percentage of methæmoglobin in the total circulating blood pigment. This is accompanied by surprisingly little other abnormality, though mild dyspnœa on exertion may be present.

Both Lian and Deeny and their co-workers treated their cases with ascorbic acid. They found that oral or intravenous administration relieved the cyanosis and converted the major portion of the methæmoglobin present into reduced hæmoglobin.

Hartmann *et al.* (1938) and Cox and Wendel (1942) obtained good results by using methylene blue in the treatment of the methæmoglobinæmia caused by sulphanilamide drugs.

Through the kindness of Professor Henry Barcroft, we have had the opportunity of studying a case of idiopathic methæmoglobinæmia successfully treated with ascorbic acid and methylene blue. Our reported observations include a few in-vitro experiments upon the patient's blood.

Case report

The subject (P. B.) was an orphan girl aged 25 years. No family history was obtainable. She stated that she had had a dark bluish complexion as long as she could remember. She was employed as a hospital domestic, and was

accustomed to fairly heavy work. She complained of no untoward symptoms, and there was no dyspnœa. When first seen on 24.8.43 she had a dusky cyanotic hue of the face and hands, particularly marked in the lips, ears and fingernails. The cardiac apex beat was slightly heaving, 4 in. from the midline ; a systolic murmur was present. The digestive system and the urine were normal.

The blood was a dark chocolate colour and contained an intracorpuscular pigment which was identified spectroscopically as methæmoglobin. No other abnormal pigment was present. The total hæmoglobin calculated from accurate iron analysis was 17·0 g. per 100 ml., *i.e.* 115 per cent. of the N.P.L. Haldane standard. The pigment capable of carrying oxygen, as measured by Van Slyke oxygen capacity, was 10·8 g. per 100 ml. The difference of 6·2 g. per 100 ml. consisted of methæmoglobin, which thus formed 36 per cent. of the total blood pigment. The R.B.C. count was 5·3 million, and reticulocytes 2·6 per cent. The mean corpuscular volume was 90 cu. μ. The white cell count was 10,000, the differential count normal.

The plasma ascorbic acid concentration was 0·25 mg. per 100 ml.—a low level, which is nevertheless within the normal range under war-time dietary restrictions.

EXPERIMENTAL

Treatment with ascorbic acid

The results of the oral administration of ascorbic acid are shown in fig. 1. On 300 mg. per day, the oxygen capacity of the blood rose
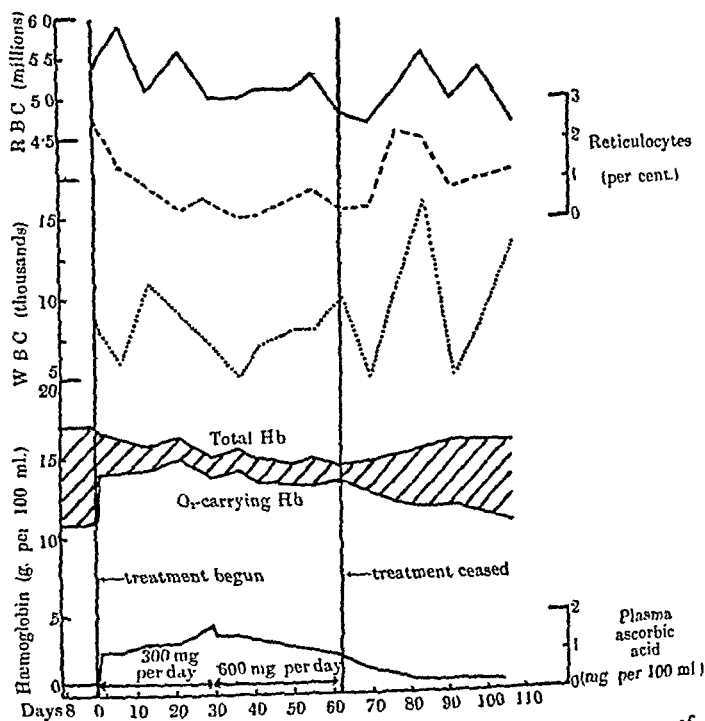
FIG. 1.—Effects of oral administration of ascorbic acid on a case of methæmoglobinæmia.

almost at once from 10·8 to 14·1 g. Hb. per 100 ml., in parallel with the immediate marked increase in plasma ascorbic acid. Cyanosis

lessened after three days and disappeared completely within a week. As treatment was continued, the total Hb. decreased slowly to 15 g. per 100 ml.; thus a residual amount of about 1 g. of met-Hb. per 100 ml. (or about 7 per cent. of the total pigment) remained in the blood. (The hatched portion on the chart indicates met-Hb.) At the same time the red-cell count fell rather irregularly to 5 million and the reticulocytes came down to about 0·5 per cent. These factors reached relatively constant values in three weeks' time, and after four weeks the dose was increased to 600 mg. per day. There were no further significant changes during a 30-day period on this dosage.

Treatment was then discontinued and a gradual reversal of the above effects was observed; the changes were detectable 10 days
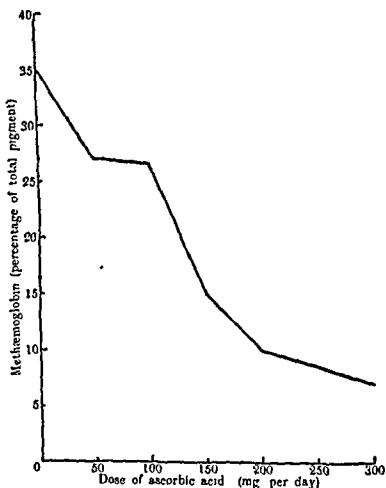
Fig. 2.—Level of methæmoglobin in patient's blood on various dosages of ascorbic acid.

after cessation of therapy, and after 30 days the oxygen capacity, total hæmoglobin and plasma ascorbic acid had very nearly regained their former values. The red-cell and reticulocyte counts rose to their original levels in three weeks; there was, however, a subsequent fall.

The changes in the white-cell count shown on the graph are somewhat irregular; on the average the patient tended to have a higher W.B.C. value while untreated than when undergoing treatment. The differential counts (not recorded) were normal throughout.

Next, varying doses of ascorbic acid ranging from 50 mg. per day upwards were tried. It was found that 200-300 mg. per day were

required to keep the amount of met-Hb. below 10 per cent. and the patient free from cyanosis. The levels of met-Hb. maintained on different dosages are illustrated in fig. 2.

Treatment with methylene blue

When the patient had returned to her original condition after cessation of ascorbic acid therapy, she was given 4 grains of methylene blue daily for three weeks. Fig. 3 shows that the effect of this treatment on the met-Hb. is much the same as with ascorbic acid. The minimal dosage necessary to maintain the met-Hb. below 10 per cent. was found to be 1 grain daily. A combination of 50 mg. ascorbic acid daily with 1 grain methylene blue every other day was also effective. Minimal methylene blue treatment has been continued for many months without any untoward effect.
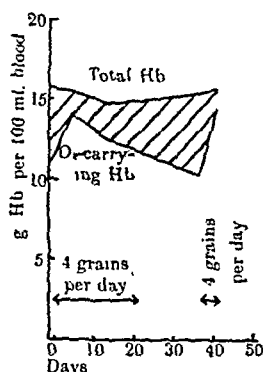
FIG. 3. — Effect of oral methylene blue on methæmoglobin level in blood. Hatched area represents methæmoglobin, i.e. the difference between total hæmoglobin and hæmoglobin capable of transporting oxygen.

In every oral dosage regime investigated, at least 7 per cent. of the blood pigment remained in the form of met-Hb. It was thought that this residual pigment might be converted to ordinary Hb. by administering methylene blue intravenously.

Two experiments to test this were performed. The patient was on an oral dosage of 4 grains methylene blue daily. In the first experiment 52 mg. (1 mg. per kilo body weight) of methylene blue in the form of a sterile 0·25 per cent. aqueous solution were given in a single intravenous injection. Samples of blood were taken after 1, 3 and 5 hours. In the second experiment an initial injection of 52 mg. was followed 2½ hours later by a further 25 mg. The results are illustrated in fig. 4. In both experiments, the amount of met-Hb. in the blood was considerably reduced ; the final level was between 0·2 and 0·4 g. per 100 ml. or about 2 per cent. of the total hæmoglobin. These figures were obtained from the difference between iron analysis and oxygen capacity. Since it has been found that a similar difference frequently exists in normal human blood (the cause of which has not been definitely determined), it cannot be stated categorically that a residue of met-Hb. remained after intravenous methylene blue. None was detectable spectroscopically in our blood samples. In addition, for such small differences, experimental errors in the estimations become increasingly significant. It is noteworthy that the extra injection of 25 mg. in expt. 2 did not alter the blood picture.

Action of therapeutic agents on blood in vitro

In the following experiments met-Hb. was determined by the method of Evelyn and Malloy (1938), or in some cases by a less precise method in which a 1 : 20 dilution of blood in water was viewed in a Klett colorimeter with a spectroscope eyepiece. Met-Hb. bands in the
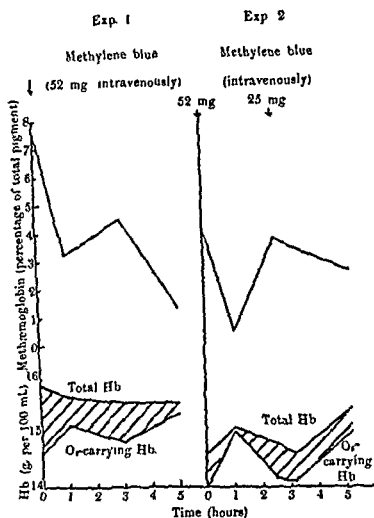
FIG. 4.—Effect of intravenous methylene blue (in addition to oral methylene blue) on methæmoglobin level.

red were matched in intensity; then all the pigment in the right hand cup was converted to met-Hb. by addition of solid ferricyanide, and the bands rematched. In a typical experiment, with bands originally matched at 30 mm., after addition of ferricyanide, match was obtained with the right hand cup at 6·0 mm. Therefore met-Hb. originally present $= \dfrac{6·0}{30} \times 100 = 20$ per cent. of total pigment.

Ascorbic acid. Lian *et al.* and Deeny *et al.* found that ascorbic acid added to samples of blood from their cases reduced the met-Hb. to Hb. We have confirmed their observations on our patient's blood.

Methylene blue. Incubation of saponin-hæmolysed saline-diluted blood from our case with methylene blue at 37° C. for one hour resulted in the disappearance of the met-Hb. A small effect was first observed at a concentration of 0·05 mg. per 100 ml. of methylene blue; the proportion of met-Hb. altered in one hour increased with the con-

centration of methylene blue until at a level of just over 0·5 mg. per 100 ml., the pigment was completely destroyed. These results are illustrated in fig. 5.
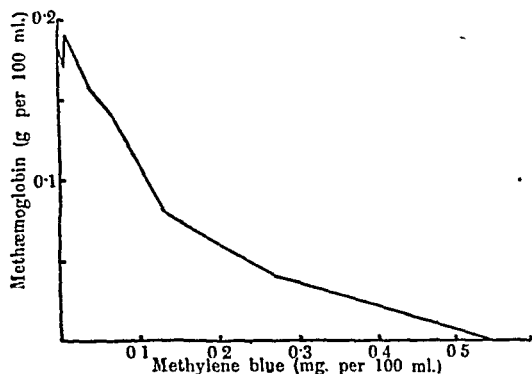
FIG. 5.—In-vitro effect of methylene blue on methæmoglobinæmic blood (4 g. met-Hb. per 100 ml. diluted 1 : 20 with normal saline : 1 hr. at 37° C.).

Glutathione. The concentration of glutathione in the patient's blood was found to be normal (66 mg./100 ml.). Incubation of the blood at 37° C. for 4 hours with added reduced glutathione in concentrations ranging from 0·01 to nearly 1 per cent. had no significant effect upon the concentration of met-Hb. present. It is possible that longer incubation would have produced a measurable reduction of met-Hb. Gibson (1943) points out that while glutathione will reduce met-Hb., its action is much less powerful than that of ascorbic acid.

Action of normal cells and plasma. (a) Several experiments were performed to test the effect upon the met-Hb. in the patient's red cells of the addition of normal blood or plasma. In general no significant effect on the rate of reduction of met-Hb. could be demonstrated by normal blood, cells or plasma.

(b) Fresh liver suspension was, however, effective in reducing the met-Hb.

(c) The addition of glucose had no effect ; lactate produced a small decrease in met-Hb., which may possibly be significant, while methylene blue and lactate produced irregular results.

(d) A sample of normal blood of the same blood group as the patient had the Hb. partially converted to met-Hb. by amyl nitrite in normal saline (Warburg et al., 1930). The cells were then spun down and the excess of amyl nitrite removed by repeated washing with normal saline. These cells were then incubated at 37° C., and washed red, cells from the patient were incubated simultaneously. Met-Hb. was estimated at intervals. The results showed very little change in the content of met-Hb. of the patient's red cells over a period of 7 hours ; in the presence of normal serum or the patient's serum, with or without added glucose and phosphate, the same slow rate was observed. On the other hand, the reduction of the artificially

created met-Hb. in normal cells took place at a much greater rate. No significant change was produced by the addition of glucose and phosphate, nor was any inhibitory effect of the patient's plasma upon the reaction observed.

DISCUSSION

The results upon our patient confirm the findings of Lian and of Deeny and their co-workers that in cases of congenital methæmoglobinæmia daily administration of ascorbic acid will relieve the cyanosis and reduce the proportion of methæmoglobin present to about 7 per cent. The maximum effect obtained with ascorbic acid would seem to be directly related to saturation ; it occurred at a dosage of between 200 and 300 mg. per day, greater amounts producing no further change. The small change in methæmoglobin produced by a dosage of 50 mg. of ascorbic acid per day did not visibly affect the cyanosis. Thus it is clear that even a liberal normal diet would not be expected to provide, in itself, sufficient vitamin C to alter the patient's clinical state.

The other changes in the blood cells are interesting. It was obvious that the patient, when untreated, had a certain degree of compensating polycythæmia, shown by a high total hæmoglobin and a tendency to a high red cell count. Both fell, and surprisingly promptly, when ascorbic acid was administered. This evidence might indicate that the factors controlling the levels of hæmoglobin and of red cells in the body are considerably more dynamic than has hitherto been believed. The raised white cell count, which also showed a tendency to fall during treatment, is not readily explainable.

Treatment with methylene blue or a combination of ascorbic acid and methylene blue was equally effective. The intravenous experiments showed that the amount of methæmoglobin could be reduced to 2 per cent. or less by the introduction of a sufficient concentration of methylene blue into the circulation. It is quite probable that no methæmoglobin remained, the difference between total iron content and oxygen capacity of the blood being of the same order as differences frequently found in normal blood. It is probable that the methylene blue is reduced to the leuco- form by some enzyme system present in the blood, and is then able to act upon methæmoglobin, reducing it to hæmoglobin.

Warburg *et al.* reported the presence in some mammalian red cells, including those of man, of an enzyme capable of reducing methæmoglobin to hæmoglobin, utilising glucose as substrate. According to Cox and Wendel methæmoglobin formed in dogs by the action of drugs is very rapidly reduced, and by a system that is independent of tissues other than the blood. They found that the concentration of glucose had no effect upon the action of this enzyme in the dog, whereas Brooks (1934-35) observed a marked catalytic action of glucose upon the reduction of methæmoglobin in the rabbit. It is

thus evident that there are marked species differences in the way in which methæmoglobin is dealt with, but that an enzyme system in the blood capable of reducing methæmoglobin is widespread. The available evidence points to the conclusion that this system is present in the erythrocytes themselves.

We have shown that the methæmoglobin in our patient's blood cells is changed only very slowly by incubation *in vitro*, and that the addition of intact normal cells or normal plasma, with or without glucose and phosphate, does not alter the picture. Amyl nitrite-produced methæmoglobin in normal cells, on the other hand, is quite rapidly reduced. No inhibition of this reaction was observed by the addition of plasma from the patient.

The most likely interpretation of these results is that the patient suffers from some deficiency in the enzyme system normally present in erythrocytes which prevents the accumulation of methæmoglobin. Recent work by Barcroft *et al.* (1945) and Gibson (1947) has gone a considerable way to elucidate the nature of the deficiency.

SUMMARY

1. Successful treatment of a case of idiopathic methæmoglobinæmia with orally administered ascorbic acid is reported. Oral methylene blue was also an effective treatment. The 7 per cent. residual methæmoglobin present during oral treatment was temporarily greatly reduced if not removed altogether by intravenous methylene blue.

2. In-vitro experiments indicate a probable deficiency in our patient of the normal erythrocyte enzyme system which reduces methæmoglobin and prevents its accumulation in the blood.

REFERENCES

BARCROFT, H., GIBSON, Q. H., HARRISON, D. C., AND MCMURRAY, J.	1945.	*Clin. Sci.*, v, 145.
BENSLEY, E. H., RHEA, L. J., AND MILLS, E. S.	1938.	*Quart. J. Med.*, xxxi, 325.
BROOKS, M. M.	1934-35.	*Proc. Soc. Exp. Biol. and Med.* xxxii, 63.
COX, W. W., AND WENDEL, W. B.	1942.	*J. Biol Chem.*, cxliii, 331.
DEENY, J., MURDOCK, E. T., AND ROGAN, J. J.	1943.	*Brit. Med. J.*, i, 721.
EVELYN, K. A., AND MALLOY, H. T.	1938.	*J. Biol. Chem.*, cxxvi, 655.
GIBSON, Q. H.	1943.	*Biochem. J.*, xxxvii, 615.
	1947.	*Ibid.*, xli (in the press).
„ „	1938.	*J. Clin. Invest.*, xvii 699.
HARTMANN, A. F., PERLEY, A. M., AND BARNETT, H. L.		
LIAN, C., FRUMUSAN, P., AND SASSIER	1939.	*Bull. Soc. Med. Hôp. Paris*, lv, 1194
WARBURG, O., KUBOWITZ, F., AND CHRISTIAN, W.	1930.	*Biochem. Z.*, ccxxvii, 245.

THE ISOLATION AND CULTIVATION OF VARIOLA VIRUS ON THE CHORIO-ALLANTOIS OF CHICK EMBRYOS

A. W. DOWNIE and K. R. DUMBELL

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(PLATES XXII AND XXIII)

SINCE Torres and Teixeira (1935) reported the successful propagation of a strain of alastrim virus through ten successive transfers on the chorio-allantois of developing hens' eggs, several reports of similar success with virus from typical cases of smallpox have appeared. Lazarus *et al.* (1937) passed a strain of variola virus through 45 successive transfers on the chick chorio-allantois and observed that the appearance of the infected membranes was typical and remained constant throughout the series. Buddingh (1938) described similar appearances produced by a strain of variola virus carried through ten passages and suggested that the egg technique might serve as a useful laboratory diagnostic procedure. Markham and Bozalis (1939) used the method successfully in five atypical cases of smallpox and showed that the virus isolated was neutralised by specific antiserum prepared in the rabbit. Nelson (1939, 1943) passed a strain isolated in America 200 times and a Chinese strain 44 times in eggs. He failed to find any evidence of change in virulence for animals towards that characteristic of vaccinia virus in either of the two strains so propagated. Irons *et al.* (1941) succeeded in isolating variola virus from four of five cases of smallpox by culture on egg membranes and commented on the differential value of the method, as varicella virus does not produce detectable lesions in this tissue. In a later paper Bohls and Irons (1942) recorded similar positive results from 18 of 27 smallpox cases and confirmed the earlier observations that the virus showed no tendency to change towards vaccinia on repeated egg passage. More recently North *et al.* (1944) recorded the isolation by egg culture of variola virus from smallpox crusts which had been kept at $-8°$ C. for over a year, and suggested that the lesions on the chorio-allantois were sufficiently distinct from those of vaccinia to enable the egg-culture technique to be used in the differential diagnosis of variola and generalised vaccinia.

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REFERENCES

BARCROFT, H., GIBSON, Q. H., HARRISON, D. C., AND McMURRAY, J.   1945.   *Clin. Sci.*, v, 145.

BENSLEY, E. H., RHEA, L. J., AND MILLS, E. S.   1938.   *Quart. J. Med.*, xxxi, 325.

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COX, W. W., AND WENDEL, W. B.   1942.   *J. Biol Chem.*, cxliii, 331.

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WARBURG, O., KUBOWITZ, F., AND CHRISTIAN, W.   1930.   *Biochem. Z.*, ccxxvii, 245.

with bacteria (frequently *Staphylococcus aureus*) a few drops of penicillin solution containing 100 units per ml. were dropped on each membrane after 24 hours, and again after 48 hours, if incubation was to be continued for 3 days.

Sections of membranes and skin from fatal cases of smallpox were stained by hæmatoxylin and eosin and, to demonstrate inclusions, by a modified Mann's method (Downie, 1939), sometimes preceded by hæmalum. Smears from the lesions of patients and from egg membranes were stained by Gutstein's method (1937) for the demonstration of elementary bodies.

INFECTION OF CHORIO-ALLANTOIS WITH VARIOLA VIRUS

Macroscopic appearance of lesions

The appearances produced on membranes infected with strains of variola virus from twenty patients have been constant, irrespective of the nature of the virus inoculum. The severity of infection naturally varies with the amount of active virus inoculated but has not varied appreciably with strains from different patients. The appearances are most characteristic after 48 or 72 hours, when the majority of the infected eggs have been examined.

When the amount of virus in the inoculum has been relatively large, as with most vesicle fluids, there is usually a central area, ranging from 1 to 3 cm. in diameter, which shows definite thickening and opacity of the membrane, with some evidence of congestion but no definite hæmorrhage. This central lesion is frequently smooth on the surface and is usually raised above the level of the normal membrane around it. Around the central area of infected membrane, isolated lesions are usually present in the form of small circular dome-shaped spots which are smooth on the surface with an opaque greyish white centre fading to a more translucent peripheral zone. These isolated spots are usually 0·5 mm. or less in diameter after 48 hours, and may attain a size of 1 mm. after 72 hours. When the inoculum is smaller, as is more likely when crust extracts are used, the central confluent lesion is usually absent and instead the small discrete lesions are found scattered over the surface of the membrane (fig. 1). On some membranes the lesions may number several hundred without any tendency to coalesce. Twenty-four hours after inoculation, isolated lesions are not usually visible to the naked eye although, when the inoculum is heavy, there may be at this time a central area slightly more opaque than the peripheral part of the membrane and showing some general congestion. After four days the lesions may appear slightly larger and more opaque than at 3 days, and later there may be some general increase in opacity of the membrane. The lesion does not usually progress further, however, and in one egg opened after 8 days, just before the chick was due to hatch, the infected tissue had been largely shed from the underlying healthy membrane and was present as an opaque greyish yellow, apparently necrotic mass about 1 cm. in diameter at one side of the exposed membrane, which otherwise appeared normal.

Histology

Sections of the infected membrane removed after 24 hours usually show in the central area well-marked dilatation of capillaries immediately under the ectoderm, which itself shows commencing proliferation. At other points there may be localised thickening of ectoderm which may be five or more cells deep. There is at this stage little cellular infiltration or proliferation in the mesoderm, although the endoderm may show commencing hypertrophy. After 48 hours, by which time a large central area of the membrane is affected, there is usually marked hypertrophy of the ectodermal layer, which may be many cells thick. There is frequently infiltration of granular leucocytes into the ectoderm and immediately underlying mesoderm. In the vessels there is often marked accumulation of leucocytes and some proliferation of vascular endothelium. The isolated lesions have increased in size and frequently show infiltration with leucocytes into and below the localised ectodermal proliferation. The lesions have a similar appearance after three days. Fig. 2 shows the appearance of isolated lesions at this stage. Swelling and degeneration of infected ectodermal cells have usually become well marked by the third day and in the area of the confluent lesion many of the ectodermal cells have become swollen, rounded off and detached from neighbouring cells; a layer of degenerated granular leucocytes and red cells may cover the surface of the lesion but hæmorrhage into the mesoderm is rarely seen. By the third day, also, whole areas of proliferated ectoderm may appear to become detached from the underlying tissue and, at other places, localised degeneration of these cells may give rise to the appearance of early vesicle formation. Leucocytic infiltration into such areas of degeneration may resemble the pustule formation seen in the skin of smallpox patients, although the isolated lesions in the egg membrane are always much smaller. By the fourth day much of the infected epithelium and leucocytic debris has been thrown off and regeneration of the underlying ectoderm has begun. At all stages, thickening of the endoderm immediately below the ectodermal lesions is to be found, and the mesoderm may also be thicker than normal as a result of the inflammatory changes described.

Inclusion material. This may make its appearance within 24 hours of infection in the form of fine acidophil granules in the cytoplasm of ectodermal cells. The inclusions are more readily seen in the central area of heavily infected membranes and may not be found at this stage in the discrete areas of ectodermal proliferation. After 48 hours the acidophil material has generally increased in amount and is to be seen as granular eosin-staining masses, irregular in shape, sometimes at one side of the nucleus, sometimes at both poles, while in other cells the nucleus may be completely surrounded (fig. 3). Occasionally the acidophil material is present in the form of several small homogeneous bodies, varying up to several μ in diameter,

PLATE XXII

CULTIVATION OF VARIOLA VIRUS

Fig. 1.—Chorio-allantoic membrane 3 days after infection with extract of crusts from a case of smallpox. × 1·5.
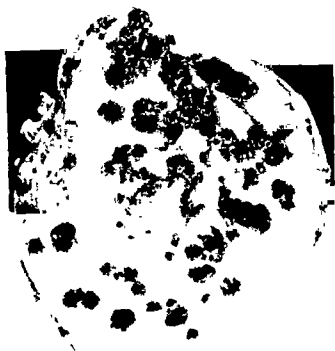
Fig. 5.—Chorio-allantoic membrane 3 days after infection with vaccinia virus from a vaccination vesicle in a young adult. × 1·5.

Fig. 2.—Section through chorio-allantois of 3-day lesions due to variola virus. Hæmatoxylin and eosin. × 40.

CULTIVATION OF VARIOLA VIRUS

Fig 3 —Section through 3 day variola lesion on chorio allantois, showing cytoplasmic inclusions in ectodermal cells. Hæmalum, eosin and methyl blue. Dufay film. ×300

Fig 4 —Section through early smallpox lesion in human skin, showing inclusion material in cells of malpighian layer. Eosin and methyl blue Dufay film ×300.

in the cytoplasm of infected cells: single large homogeneous inclusions, as depicted in the cells of the rabbit's cornea after infection with variola or vaccinia virus, have not been found. The ballooned cells, which appear in the areas of infected ectoderm by the third day, may have the cytoplasm filled with inclusion material and the nucleus may be shrunken and deeply stained. In the discrete lesions, seen in membranes infected directly with material from cases of smallpox, inclusion material is usually scanty and may be restricted to a few swollen cells in the deeper ectodermal layers in the centre of the lesion. On passage, the inclusion material appears to increase in amount and, with one strain which has been passed 16 times on eggs, acidophil material was constantly present in the ectodermal cells of discrete lesions as well as in the central confluent areas. Even in these later passages, however, the area of ectodermal proliferation appears to extend beyond the area of infection and inclusions are not as extensive in distribution as the ectodermal hypertrophy. Inclusions were not identified in mesodermal cells, nor were they found within the nuclei of ectodermal cells (*cf.* Torres, 1935-36).

Pieces of skin from six fatal cases of smallpox were fixed and stained by the same methods as those used for egg membranes. Cytoplasmic acidophil material can be seen in the infected swollen cells of the malpighian layer in vesicles and pustules (fig. 4). In some of the epithelial cells the inclusion material is present in the form of numerous small discrete bodies, while in others it appears as irregular granular masses almost filling the cytoplasm. These appearances are similar to those observed in the ectodermal cells of the infected chorio-allantois. It is likely that the finely granular material corresponds to masses of elementary bodies such as were observed by Himmelweit (1938), using the ultropak method, in the living ectodermal cells of the chorio-allantois of duck embryos infected with vaccinia virus. As noted by Hammerschmidt (1918) and others, the inclusions in the skin of smallpox cases, as in the skin of rabbits and calves infected with vaccinia virus, are unlike the Guarnieri bodies seen in the infected corneas of rabbits.

Elementary bodies can readily be found in suitably stained smears from infected egg membranes although, as noted by Lazarus *et al.*, Buddingh and others, they are not usually so numerous as in corresponding smears from vaccinia-infected membranes, nor as in properly prepared smears from papular or vesicular lesions in smallpox cases.

Survival of embryos

It has been noted by others that the embryos usually survive infection of the chorio-allantois with variola virus, although North *et al.* recorded a 50 per cent. mortality among embryos from the sixth passage onwards when a heavy inoculum was used. None of our strains has proved regularly lethal to embryos when the inoculum was

heavy. The one strain which has been passed 16 times on the chorio-allantois also failed to produce death of the embryos. On two occasions embryos were found dead 3 days after inoculation (6th and 7th passages), but in subsequent transfers the embryos regularly survived up to the time of examination (3 days). The ground membranes, when suspended in broth and titrated in 10-fold dilutions of the supernatant, usually proved infective in dilutions up to 10^7; the undiluted supernatant fluids contained, therefore, many infective doses which nevertheless failed to produce fatal infections in the embryos.

Comparison of variolar and vaccinial lesions on the chorio-allantois

By comparison with variola, the lesions produced by vaccinia virus are much larger and, after 3 days' incubation, isolated lesions may attain a diameter of 3 mm. (fig. 5). These lesions tend to be flatter and more necrotic, so that the surface is rougher and not dome-shaped like the smaller lesions of variola. Furthermore, hæmorrhage into the membrane is more frequent, especially when the lesions are confluent. Although, with egg-adapted strains of vaccinia, a heavy inoculum frequently causes fatal infection of the embryo, vaccinia virus from the human skin does not at first prove excessively virulent, so that the relative virulence of the two viruses is of no great value in diagnosis. Histologically, the vaccinial lesions are more extensive and more destructive than those produced by variola virus, and proliferation of the ectodermal cells is not such a marked feature. The inclusion material in infected ectodermal cells has a similar appearance in the two types of infection, although with variola it tends to be more coarsely granular.

Neutralisation of variola virus by antivariolar and antivaccinial sera

Neutralisation tests were made by the pock-counting method on infected egg membranes. The virus inoculum used in the tests shown in table I was the supernatant fluid obtained by centrifugation of a membrane suspension from eggs infected with vesicle fluid from a case of smallpox. The antivariolar serum was obtained from a woman aged 35 years on the 18th day of a moderately severe attack of smallpox. This patient had been vaccinated in infancy. The antivaccinial serum was obtained from a young adult male who had been successfully vaccinated for the first time 17 days previously. Normal serum used as a control was obtained from a young adult who had never been vaccinated and who subsequently gave a typical primary response to vaccination. The heat-inactivated sera were mixed with virus suspension to a final serum dilution of 1 : 4. The effect of normal serum was controlled by a 4th mixture in which

saline was substituted for serum. All mixtures were allowed to stand for one hour at room temperature and eggs were then infected with inocula of equal volume. The eggs were opened after three days, and the membranes carefully cut out and immersed in formol-saline for greater ease in counting. There was no significant difference in the number of lesions on membranes infected with saline-virus and normal serum-virus mixtures. From the results of one such experiment shown in table I it will be seen that there was a very considerable

TABLE I

Neutralisation of variola virus by human antivaccinial and by antivariolar serum

	Serum in serum-virus mixtures		
	Antivariolar	antivaccinial	Normal human or saline
No. of lesions on egg membranes . .	18, 2, 8, 4, 19, 10	34, 2, 9, 16, 22	550, 250, 245, 230, 198, 340
Average no. of lesions	10	17	302

reduction in the pock count compared with the controls when either variolar or vaccinial antiserum had been mixed with the virus. The two antisera used had comparable titres when examined by the complement-fixation test against a vaccinial antigen and there seemed to be no significant difference in their neutralising power against variola virus as tested by the pock-counting method on egg membranes.

THE DIAGNOSTIC VALUE OF THE CHORIO-ALLANTOIC
INFECTION TECHNIQUE

The successful results obtained with material from 20 cases of smallpox are summarised in table II. The cases have been arranged, not in strict chronological order, but according to the nature of the inoculum. All the cases were in adolescents or adults and three died of their infection (nos. 2, 3 and 12). The cases occurred in eight separate outbreaks. In three outbreaks infection was derived from Egypt or the Near East, in the remaining five from India. From the first 7 cases shown in table II vesicle or pustule fluid only was available ; from 4 cases fluid and crusts were examined separately ; from case 12—one of hæmorrhagic smallpox—virus was recovered from a clotted sample of blood taken two days before death as well as from vesicle fluid : from the last 8 cases listed, crusts only were examined. Specimens were collected between the 4th and 23rd day of illness, but the severity of infection of the chorio-allantois was not directly related to the stage of the disease at which the specimens were collected. In general, however, the inoculation of vesicle or

pustule fluid (diluted) produced more extensive lesions than did extracts of crusts. In case 1, the original fluid was diluted approximately 1 : 100 and 1 : 1000 for the 3rd and 4th eggs. . The

TABLE II

Isolation of virus from 20 cases of smallpox

Case no.	Day of disease on which specimen obtained	Material for test	Result of inoculation on chorio-allantois	
			No. of eggs inoculated	Lesions produced
1	6	Fluid	4	C, C, 195,* 2*
2	11	,, P.M.	4	C, C, C, C
3	13	,, P.M.	4	C, C, SC, SC
4	8	,,	2	90, 19
5	8	,,	2	C, C
6	4	,,	2	C, C
7	9	,,	2	SC, 12
8	{ 10	,,	5	C, C, C, SC, SC
	{ 23	Crusts	3	C, C, C
9	{ 10	Fluid	4	SC, SC, 150, 35
	{ 10	Crusts	3	C, C, C
10	{ 6	Fluid	2	C, C
	{ 13	Crusts	3	45, 30, 11
11	{ 8	Fluid	2	C, C
	{ 15	Crusts	3	55, 18, 9
12	{ 6	Blood	5	6, 10, 0, 0, 0
	{ 7	Fluid	3	C, C, C
13	17	Crusts	5	25, 12, 10, 6, 2
14	12	,,	4	120, 85, 14, 13
15	21	,,	2	250, 150
16	18	,,	2	6, 2
17	23	,,	2	SC, SC
18	14	,,	2	300, 220
19	14	,,	2	80, 14
20	11	,,	3	C, C, C

* = inoculated with 1 : 100 and 1 : 1000 dilutions of the vesicle fluid.

C = area of central confluent infection with peripheral discrete lesions.
SC = numerous discrete lesions tending to become confluent.
Figures indicate the number of discrete lesions on individual egg membranes.

occurrence of confluent lesions in membranes inoculated with other specimens of fluid or crust extract indicated that these inocula would probably have been infective if diluted 1 : 1000 or even higher. Where the original inoculum produced only discrete lesions, transfer to further eggs usually resulted in confluent infection of the membranes.

In two cases variola virus was not isolated by the egg-culture method. The first was in a patient who had been twice successfully vaccinated within three months of his suspected variola infection; this was diagnosed clinically on the history of a febrile attack commencing six days after the second vaccination and the appearance of one typical pustule and two doubtful lesions a few days later. Two tiny dry crusts collected on the tenth day were submitted for examination. This material was insufficient for a serological test and an extract failed to produce variolar infection on egg membranes.

The second failure was in a typical mild clinical case. An extract of crusts collected on the tenth day gave a positive serological test for variola-vaccinia antigen. This fluid, prepared by extraction of crusts with 9·0 per cent. NaCl, showed on culture numerous staphylococci and, as we had not then adopted penicillin to suppress such bacterial contaminants, it was treated with ether for eight days in the refrigerator. At the end of this period the extract was bacteriologically sterile but, after removal of the ether, it failed to produce infection on the chorio-allantois. Subsequent experience leads us to believe that inoculation of the fresh extract mixed with penicillin would have yielded a positive result.

In 14 of the 20 cases shown in table II serological tests for antigen in fluid or crusts were made and gave positive results. Two of the remainder (cases 1 and 12) were clinically typical severe cases, and smears from the vesicles showed numerous elementary bodies. In case 4, which was mild, the sample of crusts submitted was inadequate for serological test. In case 5, which showed very few lesions, the specimen submitted consisted of a little dried exudate on the tip of a pipette which had been used in an attempt to get vesicle fluid. In cases 6 and 7, only dried smears on slides were submitted for microscopical examination. In each instance the dried film on one slide was taken up in a little saline for the inoculation of eggs. In these last four cases the material submitted was inadequate for serological examination.

DISCUSSION

Our experience with the egg-culture method has convinced us that successful results can be obtained with material from the majority of smallpox cases from the late papular or early vesicular stage right through to the late crusting stage of the disease. The disadvantages of the method as a routine diagnostic procedure are, first, that a supply of fertile eggs at the proper stage of development must be kept available and, second, that at least two days must elapse before a provisional positive report can be made. A quicker result is obtained both by the examination of stained smears from early lesions and by serological examination for antigen in fluid or crusts. However, the results of the examination of smears must be interpreted with caution, particularly in the pustular stage ; the egg-culture method would provide useful confirmation in these cases. It is also particularly useful when the material submitted is inadequate for serological examination.

In the differential diagnosis of smallpox, confusion with varicella may be resolved by the use of the egg-culture method, as the virus of varicella fails to produce detectable lesions in the chorio-allantois. Vaccinial infection is readily differentiated by the macroscopic appearance of the membrane lesions. The lesions of herpes febrilis

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6	4	„	2	C, C
7	9	„	2	SC, 12
8	{ 10	„	5	C, C, C, SC, SC
	{ 23	Crusts	3	C, C, C
9	{ 10	Fluid	4	SC, SC, 150, 35
	{ 10	Crusts	3	C, C, C
10	{ 6	Fluid	2	C, C
	{ 13	Crusts	3	45, 30, 11
11	{ 8	Fluid	2	C, C
	{ 15	Crusts	3	55, 18, 9
12	{ 6	Blood	5	6, 10, 0, 0, 0
	{ 7	Fluid	3	C, C, C
13	17	Crusts	5	25, 12, 10, 6, 2
14	12	„	4	120, 85, 14, 13
15	21	„	2	250, 150
16	18	„	2	6, 2
17	23	„	2	SC, SC
18	14	„	2	300, 220
19	14	„	2	80, 14
20	11	„	3	C, C, C

* = Inoculated with 1 : 100 and 1 : 1000 dilutions of the vesicle fluid.

C = area of central confluent infection with peripheral discrete lesions.
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NORMAL AND ABNORMAL BLOOD-COUNTS ON THE WITWATERSRAND

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On the Witwatersrand several major factors may co-operate in modifying the blood-count. There is firstly the altitude, which is about 6000 feet above sea level ; secondly there are the unclouded skies, which must influence the iron content of dietetic articles and thus may materially affect the health and robustness of individual subjects ; thirdly there is the problem of deep level mining, in which a very large proportion of the male population participates. Some individuals daily descend to depths of nearly 9000 feet, and this barometric factor may combine with the possible effects of exposure to silica dust in modifying the blood standards of such workers.

This paper briefly presents the results of an analysis of the blood-count in normal subjects and in sufferers from macrocytic anæmia. None of the individuals under review had ever served as miners on the Witwatersrand.

Technique

Standards

The following standards were observed. Capillary blood only was examined ; it was alternately obtained from the lobe of the ear or from the finger pulp. The Joseph (1922) modification of the capillary tube method in use by the South African Institute for Medical Research was found not entirely reliable where samples were collected by different individuals. Emmerson (1932-33) has shown conclusively that where this method is checked against the Thoma-Zeiss pipette method by an experienced technician the differences obtained do not differ significantly. But the capillary tubes are less readily controlled than pipettes, though they apparently make collection a simple matter. Having watched many students and colleagues collecting blood, in an effort to discover the source of serious discrepancies between determinations on samples separately collected, I have come to the conclusion that the chief source of error lies in inadequate wiping of the capillary tube ends. The tubes are usually over-filled. It is not sufficiently realised what a large difference a " little " extra blood at one end of the tube may make on the final count. On the other hand, if one keeps a few dozen standardised collecting pipettes handy and in good condition, it is actually an easier procedure to collect by means of these than by using capillary tubes. About half of my samples were collected in Trenner pipettes.

Erythrocytes. For the purpose of this investigation two samples were taken from each subject on different occasions to avoid diurnal and chance fluctuations. Each sample was triple checked on Thoma-Zeiss, Levy and Spencer hæmocytometers, and the averages accepted if all six counts tallied

satisfactorily. Only those counts were accepted in which the coverslip Newton's rings persisted in full up till the end of the procedure.

Hæmoglobin. In three samples, the blood to be tested was transformed into acid hæmatin by means of N/10 HCl and compared after 30 minutes with the Spencer colorimeter supplied by the Clay Adams Co. Having checked this colorimeter against other standards my conclusion was that it is entirely reliable. Dilution was effected by means of distilled water.

Leucocytes. The white-cell count was established from samples collected simultaneously with the red cell and hæmoglobin samples, and triple checked as for the erythrocyte enumeration.

Colour index (Sahli standard). These calculations were derived from standard values, as follows :—

Normal hæmoglobin = 20·7 g. Hb. per 100 c.c., *i.e.* 120 per cent.
Sahli standard.
Normal erythrocytes = 6·0 million per c.mm.

The reasons for this will emerge when the statistical evidence is analysed. The normal colour index by this standard—designated as the Sahli standard in contradistinction to the Haldane standard, which is evidently inapplicable to the Witwatersrand area, is then 1·0—a convenient neutral point from which variations in hæmoglobin concentration per cell can be assessed.

Mean erythrocytic diameter. This was established by means of the tedious micrometer method. A satisfactory personal standard by means of the Eve halometer I found myself incapable of achieving; and the discrepancies between determinations by different operators appeared to be too large for purposes of accurate analysis. For rapid determinations, when necessary, the apparatus is useful enough, but it supplies no clue to the percentage incidence of microcytes, macrocytes and poikilocytes, facts which emerge readily when Price-Jones histograms are constructed. As to the micrometric diameters of a series of cells there can be no doubt. At least 100 were measured in each case, often more. Measurements were taken at random from different fields in the smear preparation.

Mean erythrocytic surface area. Assuming that an erythrocyte as preserved on a thin, even, Leishman-stained smear preparation is a flat circular disc of very little thickness, the mean erythrocytic surface area can be easily calculated from the mean cell diameters of the Price-Jones curve, namely,

$$M.E.S.A. = 2(\pi r^2) \text{ square microns.}$$

Hæmoglobin surface concentration index. Of the 37 per cent. of solids comprising the normal erythrocyte 95 per cent. consists of hæmoglobin, which is prevented from crystallising out through being combined in some obscure way with the water molecules composing the remaining 63 per cent. of the living cell. The remainder is constituted by the thin cell membrane and some cytoplasm rich in potassium phosphate. The hæmoglobin is distributed to the inner surface of the cell membrane. What is material is its effective distribution. Bürker (quoted from Lovatt Evans, 1941) established a biologically constant ratio of 32×10^{-14} grams of hæmoglobin per square micron of corpuscle surface. This was presumably on normal specimens. I found the hæmoglobin concentration per unit of surface area to vary significantly in the anæmias. Bürker's unit at any rate is unwieldy. Moreover, it does not take into consideration either the local standards for hæmoglobin or the total red-cell count. Both of these factors are more important than the actual fraction calculated by Bürker. A mountain goat with 10 or 14 million microcytes per cubic millimeter of blood would have an entirely different total surface area to its blood than would be the corresponding " hæmic breathing surface " in a man at sea level with only 5 million relatively large cells per cubic mm.

In order to take these factors into consideration I devised a hæmoglobin surface concentration index as follows :—

$$\text{H.S.C.I.} = \frac{\text{C.I.}}{\text{M.E.S.A.}} \times 100$$

where H.S.C.I. is the hæmoglobin surface concentration index, M.E.S.A. the mean erythrocyte surface area and C.I. the colour index on a Sahli standard ; *i.e.* normal hæmoglobin = 120 per cent. Sahli and normal erythrocytes 6·0 million per c.mm. Actually, this Sahli standard gives the same indiceal result for normal blood at the Witwatersrand as the Haldane values give for normal subjects at sea level. The H.S.C.I. here presented should therefore be directly comparable with results obtained in low-lying countries.

With the information made available by means of this index the individual cell-volume determinations usually presented appear to be redundant.

Subjects of normal blood counts

In previous reports on the normal blood-count on the Witwatersrand (Emmerson, 1932-33 ; Stammers, 1933 ; Liknaitzky, 1934-35 ; Symons, 1939) medical students and laboratory workers were studied. These are not ideal subjects for blood-counts. Medical students often immigrate from various localities and thus require to undergo adaptation to the Witwatersrand altitude They often come from selected homes. If they have not already established hard-working habits they are soon required to do so. Indeed it is doubtful whether they ever become fully acclimatised. By the time they reach the fourth year of study they are usually etiolated and often undernourished. The fact that they often attain athletic triumphs may be more a reflection of their nervous habits ; or the state of psychic determination of the medical student, which he acquires unconsciously from the *tempo* communicated by his professional teachers, may make it possible for him to secure victory over the more easy-going academicians.

Moreover, medical students do not constitute the health-seeking public. Standards should reflect the features of the latter more directly. For the purpose of this investigation my subjects were thus restricted to manifestly robust students, or else I examined the blood of healthy adults between the ages of twenty and forty, recruited from the ranks of policemen, traffic officers, 'bus or tram drivers, labourers (excluding miners), housewives, typists and teachers. All were Europeans with no history of serious past or recent illness, so far as could be determined. My record, then, is based on the study of 100 males and 50 females, all residents on the Reef for the past twenty years.

RESULTS

Normal subjects

The statistical features of normal blood-counts are shown in table I.

Erythrocytes. With the low dispersion in the red-cell counts a rough standard of 6·0 million for males and 5·5 million for females seems permissible, though values between 4·9 and 7·4 million are within the range of normality. I do not believe in the advisability of extending this possible range of healthy limits further by means of mathematical calculations. The blood has very little reserve compared with the more permanent body tissues. This is due to the

than in males. Moreover, total dispersion is small, so that histograms which have been constructed revealed a similar degree of close clustering to the modal value of 6·68 μ. Taking the smallness of these cells into consideration, the high Hb. concentration appears to be particularly significant.

Mean erythrocytic surface area. The apparent range of variation is great, but it must be remembered that these values are expressed in square microns. The actual dispersion, however, is not excessive and the mean and median averages are clustered very close to the mode. This measurement gives a more arresting indication of disturbances in the direction of either microcytosis or macrocytosis.

Hæmoglobin surface concentration index. Remarkably constant values were obtained for this index, and the dispersion is low though the range of normal limits extends from 1·13 to 2·40. Only occasional subjects furnished such extreme values. The significance of this concentration index will emerge more directly when the abnormal blood-counts are reviewed.

Leucocytes. The values obtained do not differ significantly from the averages established at coastal levels, nor did the differential white-cell count (not included in this paper) give a clue to any suggestive regional telluric adaptation. On the other hand, this correspondence of the white-cell count with normal standards elsewhere suggests that gross technical errors have not been overlooked and that the erythrocytic response is a specific adaptation independent of total formed element/plasma ratio modifications.

Macrocytic anæmias

The results statistically analysed in table III are derived from a study of 300 cases of macrocytic, hyperchromic anæmias among residents on the Witwatersrand. These values assembled in table III are particularly useful at this juncture in showing the manner in which the features of the abnormal blood-counts are influenced by the local standards recorded in table I.

Erythrocytes. Under the heading of anæmia have been included a few instances of red-cell counts of 6 million cells and over, because in these cases the hæmoglobin values were remarkably low and there were significant changes in cell size. Anti-anæmic treatment cleared up symptoms and restored the balance between cells and Hb. by decreasing the former and increasing the latter. These cases serve to link the series of abnormal counts with the normal series, but the majority revealed counts of 4·0 million and less. Dispersion with reference to the mean value of 4·04 was fairly extensive but not excessive, the majority of counts falling between 3·0 and 5·0 million. In view of the high normal count on the Witwatersrand all these cases merit treatment, and the criterion of fewer cells than 4·0 million per cubic mm. for therapy would leave large numbers of patients

unrelieved of their symptoms and augment the recruitment to the ranks of chronic obscure disorders.

TABLE III

Statistical features of macrocytic anœmias on the Witwatersrand
(altitude 6000 ft.)

Items	Range		Averages			Dispersion	
	Min.	Max.	Mean	Mode	Median	Standard deviation	Coefficient of variation (per cent.)
Erythrocytes (millions per c.mm.)	1·0	7·0	4·04	4·00	4·00	0·908	22·50
Hæmoglobin (g. per 100 c.c.)	11·9	24·7	18·0	17·8	18·23	2·283	12·69
Colour index (Hb. = 120 Sahli; R.B.C. = 6·0 million)	0·7	2·0	1·37	1·30	1·35	0·224	16·40
Mean erythrocyte diameters (linear microns)	4·0	11·0	7·8	8·0	7·5	1·29	16·70
Mean erythrocytic surface areas (sq. microns)	25·0	225·0	102·0	100·0	108·0	39·00	38·8
Hæmoglobin surface concentration index	0·80	2·80	1·06	1·35	1·20	0·337	31·8
Leucocytes (thousands per c.mm.)	5·0	40·0	15·3	7·0 } 9·0 } 23·0 }	22·0	6·60	43·10

Hæmoglobin. With a mean hæmoglobin value of 18·0 g. per 100 c.c., which is in excess of the European normal, it may at first seem preposterous to diagnose anæmia. But in some of the cases with higher-range Hb. values the red-cell counts were exceedingly low. Moreover, *cognisance must be taken of the normal values here*; and the question of effective distribution of the Hb. with reference to cell surface requires serious consideration. I have shown, in a paper to be published, that a direct correlation exists between the red-cell count and the blood-sugar level, and that an inverse relationship exists between the degree of hypoglycæmia and the Hb. value. These *correlations suggest that the portion of the blood sugar which is not readily exhausted by fasting is possibly adsorbed to the red-cell surface and may stand in close relationship to the Hb. concentration per unit area of cell surface.* This may explain the close resemblance between certain features of anæmia and hypoglycæmia. It also suggests that this reserve of blood sugar plays an obscure part in *the oxygen- and carbon dioxide-carrying functions of the blood.* With an ineffective distribution of Hb., anæmia may have to be diagnosed even in the presence of high Hb. levels. Such anæmias must then be diagnosed as hyperchromic.

Colour index. The hyperchromia of these anæmias would have to be regarded as ultra-hyperchromic if the standard for comparison

fully acclimatised to the high altitudes of the Witwatersrand, or who may be overworking; medical students in particular appear to be unsuitable and show a relatively low hæmoglobin content.

2. There is a normal relative erythrocytosis on the Witwatersrand, with a corresponding increase in Hb. level. New standards are established, namely 6 million red cells per c.mm. and 120 per cent. Hb. (Sahli) respectively. On this basis the colour index thus remains at 1·0, but if the Haldane sea level standard be used the colour index is much higher. All the advantage, however, is on the side of using these Sahli determinations as normal values.

3. The normal cell is slightly smaller in diameter and surface area than at sea level in Europe.

4. The leucocyte count is not modified through acclimatisation to the high altitude.

5. The macrocytic and hyperchromic anæmias of the Witwatersrand discussed in this paper are only explicable in terms of these new local standards. The macrocytosis is best reflected in an increased mean erythrocytic surface area. The hyperchromia is an expression of the local trend of telluric altitudinal adaptation.

6. But the most significant feature of these anæmias is the decrease in Hb. concentration per unit of erythrocytic surface area as established through determinations of the hæmoglobin surface concentration index (H.S.C.I.). The increase in cell surface area is found to be disproportionate to the increase in the colour index. Consequently microcytes have a high Hb. concentration per unit of erythrocytic surface, and in macrocytes this concentration is very sparse. It seems to be self-evident that these factors must alter erythrocyte physiology profoundly.

7. There is a slight tendency to leucopenia in afebrile anæmic cases. But there is an equally great tendency for sepsis and pyæmia to be associated with the anæmia and thus to yield moderate degrees of leucocytosis.

<div align="center">REFERENCES</div>

Emmerson, N.	1932-33. *Quart. J. Exp. Physiol.*, xxii, 369.
Evans, C. Lovatt	1941. Starling's Principles of human physiology, 8th ed., *London*, p. 526.
Foster, P. C., and Johnson, J. R.	1930-31. *Proc. Soc. Exp. Biol. and Med.*, xxviii, 929.
Joseph, F. H.	1922. *S. Afr. Med. Rec.*, xx, 282.
Liknaitzky, I.	1934-35. *Quart. J. Exp. Physiol.*, xxiv, 161.
McGeorge, M.	1936. This *Journal*, xlii, 67.
Price-Jones, C.	1931. This *Journal*, xxxiv, 779.
Price-Jones, C., Vaughan, J. M., and Goddard, H. M.	1935. This *Journal*, xl, 503.
Stammers, A. D.	1933. *J. Physiol.*, lxxviii, 335.
Symons, P. H.	1939. *S. Afr. J. Med. Sci.*, iv, 18.
Whitby, L. E. H., and Hynes, M.	1935. This *Journal*, xl, 219.

CATARRH OF THE UPPER RESPIRATORY TRACT IN MICE AND ITS ASSOCIATION WITH PLEURO-PNEUMONIA-LIKE ORGANISMS

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(PLATE XXIV)

ATTENTION has been drawn to the occurrence in mice of pleuro-pneumonia-like organisms capable of producing pneumonia (Edward, 1940). The organisms were isolated from lung lesions which followed the intranasal inoculation of sterile nutrient broth. The pneumonia was transmissible in series by the inoculation intranasally of fresh batches of mice with suspensions of the affected lungs.

After these findings were reported it was noted that a large proportion of animals in the stocks of mice under investigation were suffering from catarrh of the upper respiratory tract. Nearly all the affected mice had unilateral or bilateral otitis media and cultures from pus in the middle ears usually gave pure growths of pleuro-pneumonia-like organisms. The ætiological relationship of these organisms to the condition could not then be investigated further because of the high incidence of natural infection in the stocks of mice available for study. Lately, however, it became necessary to resume the investigation, since lung consolidation of the same type was noted during the intranasal passage of *R. orientalis* in mice, undertaken as part of the process for the manufacture on a large scale of a vaccine against scrub typhus (Buckland *et al.*, 1945). An agent capable of producing pneumonia in mice was discovered in the scrub typhus seed ; its properties were studied and it was shown to be similar to or identical with an agent present in the upper respiratory tract of mice suffering from catarrh. In the stock of mice then in use catarrh was very uncommon as a spontaneous infection and it was thus possible to investigate it and compare it with the disease described by Nelson (1937, 1942) in America, which he called infectious catarrh of mice.

TECHNIQUE AND METHODS

Swiss mice were used, most of them bred at the Experimental Field Station of the Agricultural Research Council at Compton and the rest bought from a dealer whose original stock had been obtained from Compton. There was a

very low incidence (about 1 : 500) of catarrh of the upper respiratory tract among these mice at the time they were received. In a number of control experiments at least 250 mice were inoculated intranasally with sterile nutrient broth and no lung lesions resulted. In addition 130 mice were used in control passage experiments in which mice were inoculated intranasally with broth and suspensions of their lungs passed serially by the same route into fresh groups of mice at 4- and 7-day intervals for 7 and 4 passages respectively. No lung lesions resulted.

Except where otherwise stated the mice used in these experiments were carefully chosen to ensure that they were themselves free from catarrh and came from animal boxes containing no mice with signs of the infection. The mice were used at 15-20 g. in weight. Intranasal inoculation was performed under ether anæsthesia and the dose was usually 0·05 c.c., but in the control experiments with sterile broth 0·1 c.c. was given. As a routine after inoculation mice were kept under observation for 10 days before being killed by chloroform. Their lungs were removed and examined and approximately 10 per cent. suspensions were made in a mixture of equal parts of nutrient broth and physiological saline. Suspensions were centrifuged for 3 minutes at 2000 r.p.m. and the supernatant used for inoculation of fresh batches of mice.

CATARRH OF THE UPPER RESPIRATORY TRACT AND THE PRESENCE OF A PNEUMONIA-PRODUCING AGENT

Catarrh of the upper respiratory tract in mice is readily recognised. The mice sniff and snort and frequently rub their noses and their breathing is sometimes attended by a bubbling sound which can be heard 'on auscultation. The condition does not improve, at least during observation for two to three months, nor is it fatal during this period. Otitis media, often bilateral, is a frequent complication. The disease appears to be similar to the infectious catarrh described by Nelson. Infectivity by direct contact was repeatedly demonstrated. In a typical experiment three infected mice were introduced into a population of 50 normal mice. At the end of two months all but 2 of these showed signs of catarrh, the first having become affected after about three weeks' exposure. No signs of catarrh were present in a similar control group.

Of 105 mice suffering from catarrh and inoculated intranasally with sterile broth, 36 (34·3 per cent.) developed pneumonia. In particular experiments the incidence was even higher—for example, of 14 mice recently infected by exposure to mice with catarrh, 9 (64·3 per cent.) developed lesions. These results are in marked contrast to those which followed inoculation of normal mice without signs of catarrh. As already noted, at least 250 control mice were inoculated without a single lesion. It is probable that in most of the animals the pneumonia resulted directly from the inoculation, although Nelson has shown that pneumonia may occur spontaneously as a late complication of infectious catarrh. The catarrh was a very recent infection in most of the animals used in these experiments, but in a few animals which had shown signs of catarrh for a longer time it was noted that the lung lesions looked older and might have been present before inoculation.

An agent was present in the lung lesions capable of producing pneumonia and transmissible in series. It was recovered from one lung lesion and 5 serial passages were made, each with lung suspension inoculated intranasally. Infectivity of lung suspensions was maintained during storage at $-70°$ C. The agent was also demonstrated in the snouts of mice suffering from catarrh, whether or not lung lesions had been produced by the inoculation of broth. In either case suspensions of the tissues of the nasal cavities and accessory sinuses produced pneumonia after inoculation intranasally into batches of normal mice.

PNEUMONIA-PRODUCING AGENT ISOLATED FROM SCRUB TYPHUS SEED

During the routine passage of a strain of *R. orientalis* it was occasionally noted that inoculated mice, when due for harvesting, were not as ill as usual and that their lungs showed circumscribed areas of consolidation similar to those produced by the agent present in mice with catarrh. These lesions appeared in mice inoculated with material containing comparatively few viable rickettsiæ. Suspensions of affected lungs were made in a mixture of equal parts of nutrient broth and physiological saline and passed to groups of mice immediately and after storage for three days at $+4°$ C. From material that had been passed immediately *R. orientalis* of full infectivity was recovered after a few serial passages, but repeated passage from material that had been stored in the cold showed that it had been freed of viable rickettsiæ, for in the course of 10 serial intranasal passages at 10-day intervals none of the animals died and lung lesions were circumscribed and unlike those due to *R. orientalis*. At five of these passages material was injected intraperitoneally into mice; the animals remained alive and well, free from evidence of typhus. Destruction of *R. orientalis* in the seed in this experiment by storage in the cold may have depended on a high concentration of carbon dioxide accidentally present in the refrigerator at the time. In subsequent experiments rickettsiæ were not completely destroyed by storage at $+4°$ C. for three days.

Properties of the pneumonia-producing agents

The properties of the agents isolated from mice with catarrh and from scrub typhus seed were studied. No significant differences were detected and in the following description both are considered together.

The strains were maintained by serial passage at 10-day intervals; virulence was not increased by passage at 4-day intervals. The proportion of takes among inoculated mice was irregular and ranged from 30 to 100 per cent. In addition to pneumonia the mice showed evidence of upper respiratory catarrh which was usually noticed first about the tenth day after inoculation. Catarrh was commoner in mice inoculated with the strain recovered from the scrub typhus seed.

Certain infections due to pleuropneumonia-like organisms have been favourably influenced by organic gold compounds (Findlay, Mackenzie and MacCallum, 1940; Sabin and Warren, 1940). Because the mouse pneumonia agents were believed to be pleuropneumonia-like organisms, the action of two such compounds, Lopion and Myocrisin, was tested (table II). With one exception—a mouse

TABLE II

Summary of results of chemotherapeutic experiments

Chemotherapeutic drug	Dosage and method of administration	Source of pneumonia agent	Proportion of mice with lung lesions	
			Treated mice	Control mice
Sulphathiazole	15 mg. intraperitoneally before inoculation and on 3rd and 5th days afterwards	Upper respiratory catarrh	5/12	3/11
,,	1 per cent. incorporated in food throughout experiment	,,	4/12	
,,	15 mg. intraperitoneally before inoculation and on 3rd and 5th days afterwards	Scrub typhus seed	6/6	8/8
,,	1 per cent. incorporated in food throughout experiment	,,	7/8	
Lopion	2 mg. intraperitoneally, once daily	Upper respiratory catarrh	0/9	2/9
,,	2 mg. intraperitoneally, twice daily	,,	0/8	
Myocrisin	0·1 mg. intraperitoneally, once daily	,,	1/10	4/10
,,	0·1 mg. intraperitoneally, twice daily	,,	0/10	

given 0·1 mg. of Myocrisin only once daily—the animals to which the gold compounds were administered did not develop pneumonia. However, in these experiments the proportion of untreated mice in the control groups showing lesions was unfortunately small. In another experiment 8 mice suffering from catarrh were inoculated intranasally with sterile broth and treated with 2 mg. of Lopion injected intraperitoneally twice daily. None developed lung lesions, although there were 3 with lesions in a similar inoculated group of mice with catarrh to which Lopion was not administered. These results suggest that the mouse pneumonia agent was sensitive to the two organic gold compounds tested, although to establish this more tests are required with inocula of greater infectivity.

In attempts to demonstrate immunity mice were given a series of three intraperitoneal injections (0·5, 0·5 and 1·0 c.c.) of fresh lung suspensions at weekly intervals. Ten days later they were challenged by intranasal inoculation of an undiluted infective suspension. Groups of mice were vaccinated with each agent and tested for immunity against both. The results are shown in table III. Lung

lesions occurred in the vaccinated mice, but their incidence was suggestively reduced, thus confirming the results of Pearson (1942). Unfortunately the suspensions of the agent recovered from mice with

TABLE III

Results of an experiment to produce active immunity

Source of agent	Incidence of lesions		
	Vaccinated with agent from upper respiratory tract catarrh	Vaccinated with agent from scrub typhus seed	Non-vaccinated controls
Upper respiratory tract catarrh	4/18 (22·2 per cent.)	2/19 (10·5 per cent.)	6/20 (30·0 per cent.)
Scrub typhus seed	8/19 (42·1 per cent.)	6/19 (31·6 per cent.)	12/20 (60·0 per cent.)

catarrh were of low infectivity, so that the incidence of lesions in the control group was small. This lower infectivity may also have explained the lower degree of immunity produced against this agent. The results failed to reveal any serological difference between the two agents.

PRESENCE OF INTRACELLULAR BODIES

Impression films from the cut surfaces of consolidated portions of the lungs were examined after staining by the method employed for the demonstration of *R. orientalis* (Buckland *et al.*). Later, a modification of this method suggested by Major A. Henderson-Begg, R.A.M.C., was used. After heat-fixation the film was treated with N/HCl for 2 to 3 minutes at 56-60° C. It was then stained with 1 : 2000 Unna's neutralised polychrome methylene blue for 45-90 seconds. In many of the polymorphonuclear leucocytes small stained bodies were seen. In different films the proportion of cells showing the bodies varied, and also the distinctness with which the bodies stained ; sometimes they were more obvious in one part of the film than another, but they were present in all the films examined from lung lesions resulting from the inoculation both of the agent recovered from mice with catarrh (fig. 3) and of that recovered from the scrub typhus seed (fig. 2). They were also found in consolidated lung resulting from inoculation of cultures of pleuropneumonia-like organisms isolated from lesions (fig. 1). There was no apparent difference in the appearance or arrangement of the bodies in the different types of material. The number of bodies in a cell varied : sometimes they were scanty but in other cells the whole cytoplasm was crowded with them. Frequently they were found in pairs, the distance between the paired bodies varying ; rarely they occurred in short chains. When numerous in a cell they were usually distributed throughout the cytoplasm, but they were sometimes arranged in groups. Very rarely rosettes were

found (fig. 1). The bodies were small and round; elongated bacillary elements were not seen. Occasionally larger round swollen forms were found (fig. 3). Typically the bodies were confined to poly-morphonuclear leucocytes, but occasionally mononuclear cells showed numerous granules with the appearance of these bodies. In addition to the intracellular bodies there were numerous similar bodies lying extracellularly. Cells showing these intracellular bodies were also found in films made from pus from the middle ears of mice suffering from catarrh. In such material extracellular granules were particularly numerous, but it was possible that some of these resulted from cellular degeneration.

Nelson (1937, p. 843) has stated that the infective agent responsible for infectious catarrh can be demonstrated as cocco-bacillary bodies stainable by a modification of Gram's stain. Using Nelson's staining method, in which a 1 : 4 dilution of Ziehl-Neelsen's carbol-fuchsin is applied for a few seconds as a counterstain in Gram's staining method, it was confirmed that both intra- and extracellular bodies could be demonstrated. With this stain some of the bodies were bacillary in shape. Improved results with Gram's stain were obtained by treating the films with warm acid as in the methylene blue method; this gave greater clarity to the cytoplasm.

Films were also stained by Macchiavello's method. No inclusion bodies similar to those 'produced by the mouse pneumonitis virus of Nigg and Eaton were found. The intracellular bodies already described were poorly stained and were blue.

Isolation of pleuropneumonia-like organisms

Strains of pleuropneumonia-like organisms have been repeatedly isolated from the noses and middle ears of mice suffering from catarrh and from the lung lesions produced by the two pneumonia agents.

A special medium was employed. The base was ox-heart infusion broth, adjusted to pH 8·0, with the addition of 1 per cent. peptone. For solid media 2 per cent. of agar was added. Broth or nutrient agar should not have been stored longer than 3 months. The final medium was prepared by adding to the base 10 per cent. of a freshly prepared yeast extract and 20 per cent. of horse serum and adjusting the reaction to pH 8·0. The yeast extract was made by adding 50 g. of brewer's yeast to 200 c.c. distilled water and boiling until frothing ceased. The extract was sterilised by Seitz filtration. The addition of yeast extract to the medium followed observations on the growth of pleuropneumonia-like organisms isolated from the human genital tract. Without yeast extract growth was irregular, depending on the batch of serum used, whereas its addition gave consistently good growth.

No successful isolation of these pleuropneumonia-like organisms was obtained on plates, using the same technique as that by which strains

PLEUROPNEUMONIA LIKE ORGANISMS IN MICE

Fig 1—Impression film from consolidated lung resulting from nasal instillation of a culture of pleuropneumonia like organisms Many intracellular and extracellular bodies in one cell (centre) arranged as a rosette × 1000

Fig 2—Film from lung lesion produced by an agent isolated from serub typhus seed Many pured intracellular bodes × 1000
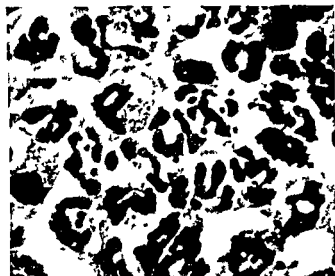
Fig 3—Film from lung lesion produced by an agent recovered from mice with catarrh Typical intracellular bodies a few of which are swollen into larger globoid bodies × 1200

from the human genital tract were regularly isolated; in particular, precautions were taken throughout to prevent drying of the plates. Isolation of pleuropneumonia-like organisms was possible only by using broth or sloppy (0·3 per cent.) agar (Beveridge, 1943) for the primary culture, although subcultures of several strains have given surface colonies on plates. This failure to obtain growth on plates in primary culture confirms my previous findings (Edward). From pus from infected middle ears and from consolidated lung tissue the organisms were isolated pure by direct inoculation of broth and sloppy agar. Pure cultures were obtained from the noses of mice with catarrh by using a selective medium containing bacteriostatics.* An alternative successful method for obtaining pure cultures from material from the nose was to make cultures from serial tenfold dilutions. Bacteria grew in the lower dilutions but frequently pure cultures of pleuropneumonia-like organisms were obtained in the higher dilutions. Broth cultures showed either a slight uniform turbidity with a fine deposit, or a nearly clear supernatant with a granular deposit. On plates the surface colonies were typical of the group of pleuropneumonia-like organisms. The strains were obligatory aerobes and in sloppy agar gave visible growth only in the uppermost centimetre of the medium. Andrewes and Welch (1946) noted that certain strains of pleuropneumonia-like organisms isolated from mice exhibited a peculiar type of motility. Two of the strains isolated in this investigation were kindly examined by Mr F. V. Welch; neither was motile.

A number of strains were tested for pathogenicity by intranasal inoculation of mice. In some of these experiments signs of catarrh of the upper respiratory tract and lung lesions resulted from the inoculation of cultures. For example, of 10 mice inoculated intranasally with the first subculture of one strain after 10 days' growth in an artificial medium, three developed catarrh and three others pneumonia. Seven days later the fifth subculture caused pneumonia in all of 9 mice inoculated, one having signs of catarrh as well. A 1:20 dilution of this subculture caused lesions in 7 out of 10 mice. The third subculture of another strain produced lung lesions in 6 out of 10 mice, one of the mice exhibiting signs of catarrh. Intranasal inoculation of the first subculture of another strain after it had been 7 days in artificial culture caused signs of catarrh in 7 and a lung lesion in one out of 10 mice. In another experiment the first subculture of a strain was without effect, but the third subculture, representing a 10,000-fold dilution of the original subculture, produced catarrh in 3 out of 10 mice, although none had lung lesions. In these experiments as well as in those in which the mouse pneumonia agents were

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* To sloppy agar and broth 1:2000 thallium acetate and about 100 units of penicillin per ml. were added. Solid media contained 1:8000 thallium acetate, and 2 drops of penicillin solution (1000 units per ml.) were spread over the surface of half the plate (Edward, 1947).

being passaged, where the proportion of mice affected by pneumonia and by catarrh was low, the mice showing signs of catarrh were usually those without lung lesions.

Discussion

Catarrh of the upper respiratory tract is common among laboratory mice ; it has been found in every one of several stocks examined. Usually it affects a high proportion of the animals, which is not surprising in view of its high infectivity. The low incidence in the stock of mice used for the present investigation was probably due to a strict policy of eradicating any mice with signs of catarrh. The condition does little to interfere with general health unless pneumonia supervenes, increasing mortality among the older animals. For most experimental purposes the employment of infected animals is thus of little consequence ; it is when mice are used for intranasal inoculation that the infection becomes important. In the present investigation, for example, when affected animals were inoculated intranasally with sterile broth more than a third developed pneumonia which was transmissible in series.

In virus investigation it is now well recognised that latent infections in the experimental animal with viruses or pleuropneumonia-like organisms are important as a frequent source of error in the interpretation of results. Several infective agents capable of producing pneumonia have been encountered in passage through mice. The presence of pleuropneumonia-like organisms is associated with one type of pneumonia (Sullivan and Dienes, 1939 ; Edward, 1940) and my present observations suggest that this is acquired from mice suffering from catarrh of the upper respiratory tract. By avoiding the use of such animals and their contacts, it is possible to escape the difficulties connected with this type of latent infection. It would be useful to discover if other apparently latent infections that have complicated virus investigations are also associated with any symptoms or signs of disease in their natural hosts. For virus research the ideal would be stocks of animals as far as possible free from all latent infections, just as uncontaminated nutrient media are essential for the study of bacteria. Stocks of mice should therefore be examined for the presence of upper respiratory catarrh and colonies built up and maintained free of the disease by eliminating all animals with signs of catarrh. Parenteral injection of organic gold compounds such as Myocrisin and Lopion may apparently prevent infection and this suggests a way in which the disease might be combated on a small scale. An attempt was made to free the strain of *R. orientalis* of the contaminating agent by a few serial passes in mice which received daily injections of these drugs. Preliminary tests suggested that this proceeding had been successful ; unfortunately the investigation had to be terminated before it could be proved that the strain of *R. orientalis* had been purified.

Contamination of *R. orientalis* with the mouse pneumonia agent probably arose from passage in mice with a high incidence of catarrh. There was no evidence that the contamination interfered with the manufacture of scrub typhus vaccine, since the agent was apparently non-pathogenic for cotton rats and did not diminish the virulence of *R. orientalis* for mice. Its presence was probably of practical importance only when the histology of the mouse lung was studied; for the intra- and extracellular cocco-bacillary bodies are easily mistaken for small forms of rickettsiæ. The histological appearances of the consolidated lung resulting from inoculation of the agent alone have already been described (Edward, 1940).

The stainable bodies in the exudates, which were demonstrated both extracellularly and within the leucocytes, resembled the cocco-bacillary bodies which Nelson regarded as the ætiological agent of infectious catarrh. Nelson obtained growth of these bodies in tissue culture but not in artificial media. In the present investigation strains of pleuropneumonia-like organisms have been isolated from infective material on numerous occasions and subcultures have reproduced the disease in mice, giving rise to both catarrh of the upper respiratory tract and pneumonia. The evidence points to these organisms as the cause of the catarrh. It is difficult to believe that a virus was also present in the cultures, persisting through several subcultures in spite of much dilution and surviving 2 weeks or more at 37° C. In one experiment there was evidence of multiplication in culture. The diminished incidence of infection that resulted from the use of organic gold compounds is suggestive confirmatory evidence, since a number of experimental diseases caused by pleuropneumonia-like organisms are susceptible to these drugs.

The finding of intracellular bodies in association with the presence of pleuropneumonia-like organisms is of interest and suggests search for similar bodies in pus cells from other conditions possibly due to pleuropneumonia-like organisms, such as non-specific urethritis in man (Beveridge). The staining method described in this paper should be of value if such a search were undertaken.

It is of interest that attempts at primary isolation of the organisms on plates failed but that culture in fluid and semi-solid media was successful, for most investigators have relied on surface plating. In any search for pleuropneumonia-like organisms in diseases of unknown ætiology, it would appear that material should always be cultured in fluid or semi-solid media as well as on plates. If bacteria are also present in the material, this will necessitate the use of appropriate selective media.

Catarrh of the upper respiratory tract is extremely common in the white laboratory rat, and Nelson (1942) has brought forward evidence that this disease is the same as or similar to infectious catarrh of mice. In exudates from the rat disease he demonstrated cocco-bacillary bodies which produced upper respiratory tract catarrh in mice, and

THE CYTOPLASMIC BASOPHILIA OF BONE-MARROW CELLS

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(PLATES XXV-XXVIII)

THE purpose of this paper is to describe observations on the cytoplasmic and nucleolar basophilia of young bone-marrow cells, utilising the cytochemical test of Brachet (1940, 1942, 1945) for ribonucleic acid. Films and sections of human marrow obtained by sternal puncture in health and in various pathological conditions form the material.

It has been clear for some time that the older division of the nucleic acids into distinctive animal and plant forms, localised in the cell nuclei (Jones, 1920a) is inadmissible. Jorpes (1928, 1934) studied the nucleic acid and nucleotide fractions isolated from the pancreas and obtained evidence for the presence of ribonucleic acid (pentose nucleic acid) as well as deoxyribonucleic acid (thymus or animal nucleic acid). More recently, extensive work by Davidson and Waymouth (1943, 1944a) in this country has demonstrated the widespread occurrence of ribonucleic acid in embryonic and adult animal tissues, particularly the former, and in rapidly growing tissues. The products of acid hydrolysis differ in the two types of nucleic acid. Deoxyribonucleic acid yields the purine bases guanine and adenine, the pyrimidine bases cytosine and thymine, phosphoric acid and 2-deoxyribose, whereas the ribonucleic acid obtained from yeast yields guanine and adenine, cytosine and uracil, phosphoric acid and the pentose, d-ribose.

The localisation of the two nucleic acids in the cell has been the object of a good deal of study and several techniques have been utilised. The Feulgen reaction has been used for the localisation of deoxyribonucleic acid within the nuclei of plant and animal cells (Feulgen and Rossenbeck, 1924), although the interpretation of the test has been criticised by Stedman (1944). Ribonucleic acid localisation has been chiefly studied by Caspersson and his school in Sweden, and by Brachet and other Belgian workers. Dempsey and Wislocki (1946) have reviewed American contributions.

Caspersson (1936, 1940) has utilised the ability of nucleic acids to absorb strongly ultra-violet light in the 2573 Å region, due to the

presence of pyrimidine rings in the nucleotides of the molecule. Using ultra-violet light with the quartz microscope, he has photographed cells in which strongly absorbing areas are considered to indicate the presence of nucleic acids. If control preparations show these areas to be Feulgen-negative, the acid is assumed to be of the ribonucleic type.

The method developed by Brachet is a cytochemical test for ribonucleic acid, depending on the power of the enzyme ribonuclease to depolymerise the acid to more diffusible nucleotides; deoxyribonucleic acid is not affected. It has been found that in sections of many tissues strongly basophilic areas are demonstrable which stain strongly with basic dyes such as toluidine blue or the pyronin of the Unna-Pappenheim pyronin-methyl green mixture (see Gatenby and Painter, 1937). Failure of such areas to stain after treatment of the section with a purified preparation of ribonuclease indicates removal of ribonucleic acid through the action of the enzyme.

Ribonuclease was discovered by Jones (1920b) but aroused little interest until the observations of Dubos and MacLeod (1937) on the presence of an enzyme in polymorphonuclear exudates which could remove the Gram-positive layer of pneumococci led to isolation by Dubos and Thompson (1938), in partially purified form, of an enzyme with the power to remove the Gram-positive staining property of pneumococci and specifically to attack ribonucleic acid. Kunitz (1940) isolated the same enzyme in pure crystalline form and investigated its properties. He found it to be a readily soluble protein of molecular weight 13,000-15,000 and able to depolymerise ribonucleic acid to readily diffusible nucleotides of lower molecular weight, but without any liberation of free phosphoric acid. The enzyme has an optimum activity at 77° C. and at pH 7·7, and possesses a high degree of heat stability, being able to withstand 3 minutes in the boiling water bath with only partial loss of activity. Dubos and Thompson found that the enzyme had no effect on a number of animal proteins or on polysaccharides. Davidson and Waymouth (1944b) found purified liver ribonucleic acid to be a specific substrate for it. There is some evidence, however, that it may be capable of attacking histones (Cohen, 1945). This last observation suggests caution in interpreting the results of the cytochemical test, particularly as Caspersson (1940) and Stedman (1944, 1945) consider histones to be important nuclear constituents, and the former author considers synthesis of histones to be an important function of the nucleolar apparatus (Caspersson and Schultz, 1940). However, it is unlikely that the basic histones will take up basic dyes strongly in the way that ribonucleoproteins appear to do.

The techniques described have provided evidence for the widespread occurrence of ribonucleic acid as a cytoplasmic constituent and often as a nucleolar constituent, particularly in young and actively growing cells. Davidson and Waymouth (1944c) have demonstrated the presence of deeply basophilic material in the

YOUNG CELLS FROM NORMAL HUMAN STERNAL MARROW

(Puncture biopsy)

Drawn from oil immersion examination

FIG 1   FIG 2   FIG 3   FIG 4
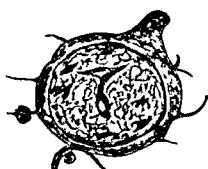
FIG 1 —Hæmocytoblast, 17 μ   FIG 2 —Myeloblast 15 9 μ   FIG 3 —Pro erythroblast, 11 5 μ
FIG 4 —Pro myelocyte, 19 2 μ   Film dried in air and stained by Jenner Giemsa   Cyto
plasmic and nucleolar basophilia indicated by shades of blue
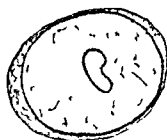
FIG 5   FIG 6   FIG 7

FIG 5 —Hæmocytoblast   FIG 6 —Pro erythroblast, 9 3 μ   FIG 7 —Myeloblast   Film wet
fixed in Susa and stained by pyronin methyl green   Cytoplasmic and nucleolar basophilic
material stained red

FIG 8   FIG 9   FIG 10

FIG 8 —Hæmocytoblast   FIG 9 —Pro erythroblast, 13 7 μ   FIG 10 —Pro myelocyte   Film
wet fixed in Susa, subjected to action of ribonuclease and stained by pyronin methyl green
Loss of basophilia shown by failure of pyronin to stain cytoplasm and nucleoli   Methyl
green still stains the nuclear and nucleolus associated chromatin

FIG 11   FIG 12   FIG 13

FIG 11 —Hæmocytoblast 9 9 μ   FIG 12 —Pro erythroblast, 9 3 μ   FIG 13 —Hæmocytoblast
FIGS 11 and 12 —From film wet fixed in Susa and stained by pyronin methyl green after
1 hr at 37° C in water at pH 7 8   FIG 13 —From similar film subjected to ribonuclease
for 1 hr at 37° C and pH 7 8, showing the loss of basophilic material
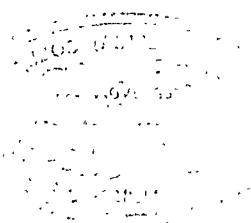
PLATE XXVI

FIG. 14.—Hæmocytoblast. FIGS. 15 and 16.—Pro-erythroblasts. FIG. 17.—Pro-myelocyte. FIG. 18.—Myeloblast. Film wet-fixed in Susa, stained by pyronin-methyl green after 1 hr. at 37° C. in water at pH 7·8. Cytoplasmic and nucleolar basophilic material stains with the pyronin.

FIGS. 19 and 20.—Hæmocytoblasts. FIG. 21.—Pro-erythroblast. FIG. 22.—Myelo-blast. FIG. 23.—Early erythroblast. Film similar to above, but subjected to action of ribonuclease for 1 hr. at 37° C. and pH 7·8. Nuclear and nucleolus-associated chromatin stains with methyl green, but the cytoplasm and central areas of nucleoli no longer stain with pyronin.

FIG. 24.—Film wet-fixed in Susa and stained by pyronin-methyl green. Hæmocytoblast (on right) and megaloblasts show cytoplasmic and nucleolar basophilia.

FIG. 25.—Similar film heated at 37° C. in water at pH 7·8 prior to staining. The baso-philia is reduced but still evident.

FIG. 26.—Similar film subjected to action of ribonuclease for 1 hr. at 37° C. and pH 7·8 prior to staining. The hæmocytoblast (upper left corner) and megaloblasts now show loss of basophilic material from cytoplasm and nucleoli. The annular nucleolus-associated chromatin stains with methyl green.

YOUNG CELLS FROM NORMAL HUMAN STERNAL MARROW
(Puncture biopsy)   ×1160

Fig 14   Fig 15   Fig 16   Fig 17   Fig 18

Fig 19   Fig 20   Fig 21   Fig 22   Fig 23

CELLS FROM THE MARROW IN PERNICIOUS ANÆMIA
(Puncture biopsy)   ×580

Fig 24
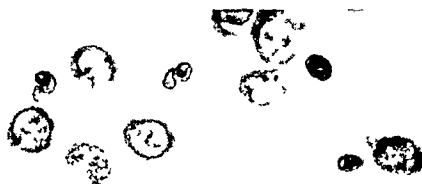
Fig 25

Fig 26

SECTIONS OF NORMAL HUMAN MARROW

(Sternal puncture biopsy)

Susa fixative   Sections cut at 3μ ×1150

FIG 27 —Pyronin methyl green stain after 1 hr in water at 37° C and pH 7 8 A hæmocytoblast in the stroma shows deep basophilia of cytoplasm and nucleoli The chromatin of adjacent erythroblasts is densely stained by methyl green

FIG 28 —Pyronin methyl green stain after washing in cold tap water Two pro erythroblasts show cytoplasmic and nucleolar basophilia and increasing nucleolus associated chromatin
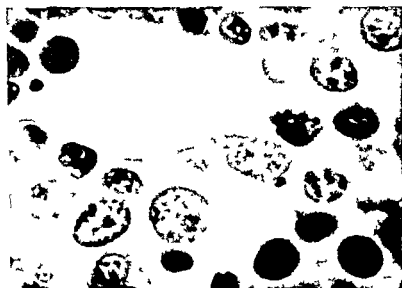
FIG 29 —Pyronin methyl green stain after the action of ribonuclease for 1 hr at 37° C and pH 7 8 A hæmocytoblast shows complete loss of basophilia from cytoplasm and centre of nucleolus A fine ring of nucleolus associated chromatin stains with methyl green Nuclei of erythroblasts unaffected

PLATE XXVIII

SECTIONS OF MARROW IN PERNICIOUS ANÆMIA

(Sternal puncture biopsy)

Susa fixative   Sections cut at 3 μ   ×1150

FIG 30

FIG 31

FIGS 30 and 31—Hæmocytoblasts and megaloblasts, with pronounced cytoplasmic and nucleolar basophilia   Pyronin methyl green stain
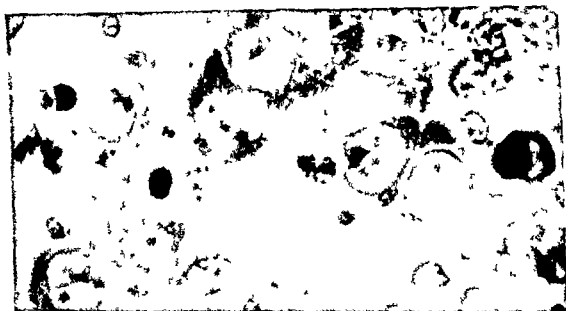
FIG 32—Similar section, stained by pyronin methyl green after 1 hr in water at 37° C. and pH 8 0   A group of megaloblasts is seen developing from hæmocytoblasts   The basophilia is reduced by the buffered water

FIG 33—A similar section stained by pyronin methyl green after the action of ribonuclease for 1 hr at 37° C

cytoplasm of parenchymal liver cells of the human fœtus and adult rat which is removable by the action of crystalline ribonuclease. Brachet (1942) has described the results of his cytochemical test as applied to the red bone marrow of various mammals and amphibians, and finds, with the pyronin-methyl green stain, that myeloblasts and erythroblasts possess strongly basophilic cytoplasm, the basophilia being lost after the action of ribonuclease. He also describes the nucleoli of the young cells as basophilic, but as losing this property after the enzyme treatment. He considers the results to indicate the presence of ribonucleic-acid concentrations in the cytoplasm and nucleoli of the young cells and that, as development proceeds, the acid diminishes considerably in amount. He finds that, in general, a high degree of cytoplasmic basophilia and a high ribonucleic acid content are characteristic of young and actively growing cells, and believes that this embryonal character closely relates the ribonucleic acid to capacity for cell proliferation and protein formation.

Thorell (1944) has carefully studied the bone marrow in rats, using the ultra-violet quartz microscope technique of Caspersson, and finds evidence for the presence of large quantities of " ribose nucleotides " in the cytoplasm of young members of the red cell and granulocyte series. He also finds the nucleoli to contain much " ribose nucleotide ", which diminishes as maturation proceeds, with accumulation of Feulgen-positive nucleolus-associated chromatin in their neighbourhood ; the disappearance of nucleoli occurs earlier in the red cells than in the granulocytic series. This author, and Caspersson and Schultz, regard the nucleolus-associated chromatin as of great importance in the synthesis of cytoplasmic ribonucleoproteins, and responsible for the accumulation of histones in the nucleolus. They consider it likely that the nucleolus-associated chromatin is similar to or identical with the heterochromatin of Heitz (1929), formerly thought to be inert. The nucleoli are developed to a maximal extent in the youngest cells and with subsequent maturation rapidly disappear, leaving a Feulgen-positive area at their site.

Dustin (1942, 1944) has studied the reticulocytes of mammals, the punctate basophilia of red cells in lead poisoning and amphibian erythrocytes, and finds the basophilic material in these cells to contain ribonucleic acid. Van den Berghe and Hoffman (1945) have reported the presence of ribonucleic acid in the granules of polymorphonuclear leucocytes.

Material and methods

Specimens of red marrow were obtained by sternal-puncture biopsy from 6 individuals in normal health and 19 patients with various pathological conditions namely :—

6 normal individuals (2 males, aged 18 and 48 ; 4 females, aged 23, 30, 31 and 35).

2 cases of idiopathic microcytic hypochromic anæmia (females, aged 44 and 49), sternal biopsies were performed before and after iron therapy.

2 cases of microcytic hypochromic anæmia secondary to chronic hæmorrhage (male, aged 30 ; female, aged 38).

1 case of normocytic normochromic anæmia of pregnancy, with pyelitis (female, aged 20).

1 case of familial hæmolytic anæmia (female, aged 24).

1 case of pan-myelophthisis (male, aged 66).

1 case of leuco-erythroblastic anæmia (female, aged 61).

3 cases with miscellaneous conditions in which erythropoiesis was of normoblastic type :—1 case of pyrexia, under investigation (male, aged 13) ; 1 case of infectious mononucleosis (male, aged 7), tibial puncture performed ; 1 case of hilar lymphadenopathy, under investigation (female, aged 42).

1 case of chronic myeloid leukæmia (female, aged 74).

7 cases with pernicious anæmia and other conditions with megaloblastic hyperplasia of the bone marrow :—2 cases of advanced pernicious anæmia (males, aged 57 and 76), sternal biopsies before and after parenteral therapy by purified liver extract in both cases ; 1 case of pernicious anæmia in relapse (female, aged 75), sternal biopsies before and during therapy by a crude liver extract ; 1 very early case of pernicious anæmia (female, aged 56), sternal biopsies before, during and after oral therapy by folic acid ; 1 case of very advanced pernicious anæmia (male, aged 63), sternal biopsies before, during and after oral therapy by folic acid ; 1 case of tropical sprue with macrocytic megaloblastic anæmia (male, aged 35), sternal biopsies at onset of and during therapy with yeast extract ; 1 case of macrocytic megaloblastic anæmia of pregnancy (female, aged 29), sternal biopsy before and after parenteral therapy by purified liver extract.

In each case films and sections were used. An air-dried film stained by the Jenner-Giemsa method aided recognition of cell types. Freshly made wet films were placed immediately in Susa fixative (Heidenhain, 1916, quoted by Romeis, 1932) and allowed an optimum of three hours' fixation. Three films (a, b, c) were taken in each case and treated as follows :—

(1) All three films were washed, treated with Gram's iodine and hypo to remove mercury, and well washed in running tap water.

(2) Film (a) was left in tap water. Film (b) was placed in a solution of purified ribonuclease maintained at 37° C. for 1 hour ; the ribonuclease was dissolved in distilled water to give a concentration of about 0·5 mg. per ml. and the solution buffered to pH 7·8. Film (c) was placed in distilled water buffered to pH 7·8 and maintained at 37° C. for 1 hour as a control.

(3) The three films were then thoroughly washed in running tap water, then with distilled water, and stained for 1 hour in a variation of the Unna-Pappenheim solution of the following composition :

Pyronin G or Y (Grübler) . . .	. 0·3 g.
Methyl green (Grübler)	. 0·7 ,,
Glycerin	. 20 c.c.
Absolute ethyl alcohol	. 2·5 ,,
0·5 per cent. phenol in distilled water to .	. 100 ,,

The constituents are mixed in a mortar to dissolve them and the solution boiled for 2 minutes and filtered.

(4) Films were then rinsed in distilled water, blotted, taken rapidly through absolute alcohol, cleared in xylol and mounted in Gurr's neutral balsam.

Films dried in air prior to Susa fixation were found less satisfactory for the preservation of cellular detail and more resistant to the action of ribonuclease, particularly in the case of the erythroblasts.

Histological sections were prepared from the marrow samples by a method recently described (White, Baker and Griffin, 1946), but tissues were fixed

initially in Susa solution for 1 hr., centrifuged, dehydrated in the alcohols and treated with chloroform ($\frac{1}{2}$-1 hr.), then post-fixed (Leach, 1945) in methyl alcohol for 12-16 hrs. before proceeding with double embedding in celloidin and paraffin.

Three sections were taken in each case and treated in exactly the same way as the films. The cytoplasmic basophilia of the marrow cells, particularly in the youngest forms, was revealed by clear red staining by the pyronin in the films and sections left in tap water. The nucleoli of the young cells also stained bright red with the pyronin. In the control films maintained at 37° C. in water at pH8, a diminution of cytoplasmic and nucleolar basophilia was usually apparent. In the films and sections treated with the enzyme, however, cytoplasmic and nucleolar basophilia was either completely abolished or very considerably reduced, the reduction being in all cases much greater than in the controls (figs. 5-33).

Susa fixative gave the best results in these preparations, and the pyronin-methyl green mixture of the composition described gave the optimum brilliance of staining to basophilic material. Formol-bichromate, Schaudinn, Zenker-formol and methyl alcohol-formol fixatives gave indifferent results, but the fixative described by La Cour (1944) was quite successful.

The purified ribonuclease was prepared from fresh ox pancreas by the method of Kunitz; the product was readily soluble in water, and salt-free. It readily attacked solutions of yeast ribonucleic acid at the hydrogen ion concentrations utilised in these experiments, the precipitability of the acid by N/10 hydrochloric acid being very greatly reduced after incubation for 1 hr. at 37° C., while control tubes with water at the same pH showed no change in the precipitability of the acid. Deoxyribonucleic acid prepared from ox spleen was not attacked by the enzyme. It was further found that the action of the enzyme on yeast nucleic acid and its efficacy in removing the basophilic components of the marrow cells was only slightly reduced by heating the enzyme for 3 minutes in the boiling water bath at pH 4·5, treatment which would certainly destroy most enzymes. After the action of the enzyme, the methyl green still stains the nucleus, and little change is revealed in this structure apart from the failure of the nucleolus to take up pyronin. Brachet (1942) regards methyl green as an elective stain for deoxyribonucleic acid in chromatin. In view of the clear-cut removal of the basophilic areas without effect on other structures by the enzyme (even after heating the enzyme at 100° C.), it appears very probable that these areas contain ribonucleic acid.

The cytochemical tests were carried out at a number of hydrogen ion concentrations between pH 6·0 and pH 8·0, the most clear-cut results being obtained between pH 7·2 and 7·8. The diminution of cytoplasmic basophilia in the controls heated at 37° C. in water has been noted by Brachet (1942), and he states that it varies in different tissues. The effect may be due to the solubility of ribonucleic acid or ribonucleoproteins in slightly alkaline media, but it is always much less marked than the result of the enzyme action.

No advantage was found in prolonging the period of enzyme action over 1 hour, loss of basophilia being maximal in this time and preservation of cytological detail good. (See addendum, p. 234.)

Results

The majority of parenchymal cells of human red marrow are developing red cells or granulocytes, with characteristic hæmo-globinisation or granulation of the cytoplasm. A small number of cells are regularly found which are the earliest recognisable members of these two series—the pro-erythroblasts and the myeloblasts

respectively (Rastelli, 1943). Both cell types have deeply basophilic cytoplasm, and in addition a smaller number of rather large, basophilic cells are found which cannot be definitely assigned to either series—the hæmocytoblast (Maximow, 1927; Turnbull, 1936; Ferrata and Fieschi, 1940, p. 10) or myeloblast of Downey (1938, p. 1963). The identity of the hæmocytoblast has been hotly debated on many occasions, and the unitarian schools of hæmopoiesis regard it as the stem cell for both red cells and granulocytes (see Downey).

The cytoplasm of hæmocytoblasts, pro-erythroblasts and myeloblasts is deeply basophilic, and in Susa-fixed, pyronin-methyl green-prepared films of marrow stains a deep red, with a variable paler pērinuclear zone (figs. 5-7). In Romanowsky-stained preparations granules are absent from the cytoplasm of the first two cells; in the myeloblast a few fine azurophil granules appear as the cell develops to the myelocyte stage (figs. 1-4). The pro-erythroblast is usually the most intensely basophilic of all marrow cells apart from the Marschalko plasma cells, but as development proceeds through the erythroblast stages, reduction in cell size occurs, hæmoglobin accumulates in the cytoplasm and the basophilia diminishes considerably (paradoxical phenomenon of Ferrata). The myeloblast acquires azurophil granules as it develops into the myelocyte, and later the specific granules appear, with rapid and great reduction in cytoplasmic basophilia.

The nuclei of the young basophilic cells contain distinct nucleoli which are also basophilic. They are difficult to discern in air-dried Romanowsky-stained films, but can be seen very clearly in the films of marrow fixed immediately in the wet state in Susa fixative, or in sections of marrow similarly fixed and stained by the Unna-Pappenheim methyl green-pyronin mixture (figs. 1-7, 27 and 30-33). The nucleoli of the hæmocytoblast are seen as 3-5 rounded structures which stain deeply with the pyronin, and there is little surrounding chromatin staining with the methyl green.

The pro-erythroblast nucleoli stain deeply with pyronin but are slightly reduced in size, and chromatin staining deeply with the methyl green is accumulating around them (figs. 4, 6, 9, 12, 15, 16, 21 and 28). As the pro-erythroblast develops to the early erythroblast, hæmoglobinisation and reduction of cytoplasmic basophilia commencing, the basophilic nucleoli rapidly disappear, and the deeply staining nucleolus-associated chromatin which has formed on their site becomes obscured by the heavily staining chromatin masses which condense in the shrinking nucleus.

The nucleoli of the myeloblast are distinctly basophilic, and only a little deeply stained nucleolus-associated chromatin is formed in their vicinity (figs. 17, 18 and 22). As the granulated myelocyte develops, however, the nucleoli gradually disappear, and more distinct, moderately deeply stained chromatin persists at their site, while remaining more discrete from the rest of the nuclear chromatin than in the red-cell series (figs. 10 and 17). The cytoplasmic basophilia

of the myelocytes is also much reduced, and is seen as a granular remnant staining pink with the pyronin.

In the normal marrow preparations, the early cells described are few in number but regularly present (figs. 14-23 and 27-29). In iron-deficiency anæmia there is often a considerable increase in the number of pro-erythroblasts, and their densely basophilic character, together with reduced basophilic nucleoli closely associated with chromatin deeply stained by methyl green, can be well seen in the Unna-Pappenheim-stained preparations. Erythropoiesis is of the normoblastic type in iron-deficiency anæmia, whether idiopathic or associated with chronic hæmorrhage.

Several cases of pernicious anæmia in an untreated state or in relapse have been examined, as well as related megaloblastic anæmias in tropical sprue and in pregnancy; they have shown marked megaloblastic proliferation in addition to a moiety of red cells developing along normoblastic lines. The megaloblasts and erythroblasts have shown cytoplasmic basophilia decreasing inversely with the degree of hæmoglobinisation. The granulocytic series have similarly shown giant bizarre forms of the metamyelocytes and pro-leucocytes in addition to normally maturing forms, and the cytoplasmic basophilia has been found to decrease very rapidly up to and beyond the myelocyte stage, the basophilic nucleoli at the same time disappearing. In addition to the cells which are definitely assignable to the red-cell or granulocyte series, whether normal or abnormal, hæmocytoblasts have been regularly found to be numerous in pernicious anæmia, and to possess similar cytoplasmic and nucleolar properties towards the pyronin-methyl green stain as the corresponding cells in normal marrows or other conditions with the normoblastic type of erythropoiesis (figs. 24-26 and 30-33). Further, in pernicious anæmia marrow it is often possible to trace intermediate forms between the hæmocytoblasts and reticulum cells of the hæmo-histioblast type of Ferrata. The latter cells have very slight cytoplasmic basophilic and one or two small basophilic nucleoli, and, in the intermediate types, cytoplasmic basophilia, and size, number and basophilia of nucleoli are increased.

In all conditions the films or sections treated with ribonuclease show similar results. Cells in the control slides with cytoplasmic or nucleolar basophilia lose this property after the action of the enzyme. The controls incubated in water at 37° C. and at the appropriate pH show some reduction over those simply washed in cold tap water, but the enzyme-treated preparations lose the property to a significantly greater extent, the general structure and the detail of nuclear chromatin staining with methyl green remaining the same.

Discussion

The results with normal human marrow and marrow of pathological states in which there is a normoblastic type of erythropoiesis support the work of Brachet (1942) and Thorell (1944) on the occurrence of ribonucleic acid as an important cytoplasmic and nucleolar constituent of cells of the marrow parenchyma and particularly of the youngest members of the red-cell and granulocytic series. The technique used in this work is similar to that of Brachet, and the validity of the results is dependent upon the specificity of the enzyme ribonuclease. The preparation used here readily attacked ribonucleic acid, and its properties have been described. Danielli (1946) has recently criticised the use of enzyme tests such as this on the ground that even the purest preparations may not be entirely specific for one substrate, and further, that removal of a cell constituent by enzyme action does not prove it to bulk largely in the composition of the part ; nor does failure to react exclude the possibility of the substance being present in a protected state.

He similarly criticises the ultra-violet-absorption technique as used by Thorell in his work, in that only precise demonstration of the absorption spectrum of a cellular constituent *in situ* can definitely prove its presence. Thorell obtained additional evidence from micro-incineration experiments, however. Brachet (1942) points out the highly elective removal of only basophilic material by ribonuclease, other cell structures remaining intact. Ribonuclease may have some action on histones (Cohen), but it is unlikely that these will take up basic dyes strongly.

With the above reservations in mind, it appears that ribonucleic acid is an important constituent of the cytoplasm and nucleoli of the hæmocytoblasts and of the youngest members of the red-cell and granulocytic series, and that this concentration corresponds with the most active period of these cells. The hæmocytoblast has well-developed nucleoli which are strongly basophilic, and if this cell develops along the red-cell series, the pro-erythroblast has often even more basophilic cytoplasm ; the nucleoli rapidly disappear, leaving accumulated nucleolus-associated chromatin, and from that point maturation is accompanied by increasing hæmoglobin accumulation in the cytoplasm and diminishing basophilia.

Similarly, if the hæmocytoblast develops along the granulocytic series, cytoplasmic basophilia rapidly declines with the appearance of the azurophil and specific granules in the cytoplasm, the nucleolar basophilia persisting however to the myelocyte stage, with gradual diminution up to that point and accumulation of paler-staining nucleolus-associated chromatin than in the red-cell series.

The developmental stages from hæmocytoblast to either red cell or granulocyte are therefore marked by a decline in the ribonucleic acid content of nucleoli and cytoplasm, the accumulation of nucleolus-

associated chromatin, and the appearance of either hæmoglobin or the highly active enzyme-containing specific granules in the cytoplasm.

La Cour has described the decrease in the Feulgen-negative nucleoli of young marrow cells with development and the accumulation in their place of Feulgen-positive heterochromatin, which appears to correspond with the nucleolus-associated chromatin of Thorell; this can be seen to take up methyl green strongly. It seems probable that the ribonucleic acid of nucleolus and cytoplasm, together with the nucleolus-associated chromatin, is closely related to the proliferation of the young marrow cells and to synthesis of their characteristic cytoplasmic contents—the chromoprotein hæmoglobin and the complex enzyme systems of the specific granules. A unique feature of the marrow cells is that loss of their power to divide is accompanied by attainment of the free state, and they end their life span as highly specialised elements.

In the bone marrow of untreated or relapsed pernicious anæmia, the accumulation of cells with strongly basophilic cytoplasm is very striking (figs. 24-26 and 30-33). Some of these cells are basophilic early erythroblasts or early members of the abnormal megaloblastic series of red cells, whilst others resemble the hæmocytoblasts of normal marrow but are increased in numbers. La Cour has studied the human marrow in health and in pernicious anæmia. He considers that a breakdown in the mitosis of early cells may account for the formation of the megaloblasts and bizarre metamyelocytes, and that competition for the supply of nucleic acids occurs between the two series at the expense of the latter. The accumulation of hæmocytoblasts and early members of the normoblastic and megaloblastic series of red cells, all with a high cytoplasmic content of ribonucleic acid, appears to be a significant feature of the marrow in this disease and in other megaloblastic anæmias.

It is noteworthy that the action of hæmopoietic substances in the megaloblastic anæmias results in a rapid return of the marrow to entirely normoblastic erythropoiesis, with maturation of basophilic cells, disappearance of the megaloblastic series and reduction in the numbers of hæmocytoblasts and basophilic early members of the normoblastic series. In the cases of pernicious anæmia studied, disappearance of megaloblasts and reduction of basophilic elements in the marrow were essentially similar, whether the hæmopoietic substance given was highly purified liver principle or cruder liver extract, or orally administered folic acid. The changes in bone-marrow morphology in the case of tropical sprue with macrocytic megaloblastic anæmia treated by yeast extract, and in macrocytic megaloblastic anæmia of pregnancy treated by highly purified parenteral liver extract, were also exactly parallel to those in pernicious anæmia.

Finally, with regard to the form in which ribonucleic acid occurs in the cytoplasm and nucleoli of marrow cells, little can be said from

the use of ribonuclease alone, which simply indicates the presence of the acid. There is a good deal of evidence, however, that it occurs in a phospholipin-ribonucleoprotein complex, particularly in the mitochondria and microsomes of various tissues (Claude, 1943; see Davidson, 1945). Brachet (1945) considers the cytoplasmic particles to be ideal centres for protein synthesis. Caspersson and Schultz believe the nucleolus and its associated chromatin to be concerned with histone formation and indirectly with cytoplasmic protein synthesis. Thorell has developed this conception in respect of the marrow cells.

With regard to the marrow specimens studied, the basophilic material was often observed to be granular, particularly in sections, and often distributed rather patchily in the neighbourhood of the nuclear membrane. Preparations fixed in mercuric acetate and stained with acid fuchsin and metanil yellow (Gough and Fulton, 1929) showed the strongly basophilic types of cells to possess cytoplasm laden with mitochondria.

Conclusions

1. Application of the Brachet cytochemical test to normal and pathological human bone marrow shows the presence of ribonucleic acid in the cytoplasm and nucleoli of young hæmopoietic cells, this substance being responsible for the basophilia of these elements.

2. The nucleolus is best developed in the hæmocytoblasts. Development to the granulocytic or red-cell series is accompanied by diminution and disappearance of the nucleolus and the development of nucleolus-associated chromatin.

3. The nucleolus has disappeared by the early erythroblast stage in the red cell series, with marked associated chromatin development. The nucleolus persists to the myelocyte stage in the granulocytic series, with finer associated chromatin development.

4. Cytoplasmic basophilia is most marked in the hæmocytoblasts and earliest members of the granulocytic and red-cell series, and particularly in the pro-erythroblasts.

5. In the red-cell series cytoplasmic basophilia diminishes gradually with the development of hæmoglobin. Rapid diminution occurs in the granulocytic series with the appearance of the granules of the myelocytes.

6. The ribonucleic acid content of nucleolus and cytoplasm in the young marrow cells and the development of nucleolus-associated chromatin appear to be closely related to capacity for cell proliferation and the development of specific cytoplasmic constituents.

7. In pernicious anæmia and other megaloblastic anæmias the characteristic megaloblastic proliferation proceeds *pari passu* with reduced normoblastic erythropoiesis, and early cells of both these series, as well as hæmocytoblasts, are prominent, their deeply basophilic cytoplasm laden with ribonucleic acid.

REFERENCES

BRACHET, J.	1940.	*C.R. Soc. biol.,* cxxxiii, 88.
,,	1942.	*Arch. Biol., Paris,* liii, 207.
,,	1944.	*Embryologie chimique, Liège,* pp. 21 and 71.
CASPERSSON, T.	1936.	*Skand. Arch. Physiol.,* lxxiii, suppl. no. 8, 73.
,,	1940.	*J. Roy. Micr. Soc.,* lx, 8.
CASPERSSON, T., AND SCHULTZ, J.	1940.	*Proc. Nat. Acad. Sci., Wash.,* xxvi, 507.
CLAUDE, A.	1943.	*Science,* xcvii, 451.
COHEN, S. S.	1945.	*J. Biol. Chem.,* clviii, 255.
DANIELLI, J. F.	1946.	*Nature,* clvii, 755.
DAVIDSON, J. N.	1945.	*Edinb. Med. J.,* lii, 344.
DAVIDSON, J. N., AND WAYMOUTH, C.	1943.	*Nature,* clii, 47.
,, ,, ,, ,,	1944a.	*Nutr. Abstr. Rev.,* xiv, 1.
,, ,, ,, ,,	1944b.	*Biochem J.,* xxxviii, 375.
,, ,, ,, ,,	1944c.	*Proc. Roy. Soc. Edinb.,* pt. 1, sect. B, lxii, 96.
DEMPSEY, E. W., AND WISLOCKI, G. B.	1946.	*Physiol. Rev.,* xxvi, 1.
DOWNEY, H.	1938.	Handbook of hematology, *New York,* vol. iii, p. 1963.
DUBOS, R. J., AND MacLEOD, C. M.	1937.	*Proc. Soc. Exp. Biol. and Med.,* xxxvi, 696.
DUBOS, R. J., AND THOMPSON, R. H. S.	1938.	*J. Biol. Chem.,* cxxiv, 501.
DUSTIN, P., Jr.	1942.	*Sang,* xv, 193.
,,	1944.	*Arch. Biol., Paris,* lv, 285.
FERRATA, A., AND FIESCHI, A.	1940.	*Emopatie acute, Florence,* p. 10.
FEULGEN, R., AND ROSSENBECK, H.	1924.	*Z. physiol. Chem.,* cxxxv, 203.
GATENBY, J. BRONTE, AND PAINTER, T. S.	1937.	Bolles Lee's The microtomist's vademecum. 10th ed., *London,* para. 320.
GOUGH, J., AND FULTON, J. D.	1929.	This *Journal,* xxxii, 765.
HEITZ, E.	1929.	*Ber. Dtsch. bot. Ges.,* xlvii, 274.
JONES, W.	1920a.	Nucleic acids, 2nd ed., *London,* p. 9.
,,	1920b.	*Amer. J. Physiol.,* lii, 203.
JORPES, E.	1928.	*Acta med. Scand.,* lxviii, 503.
,,	1934.	*Biochem. J.,* xxviii, 2102.
KUNITZ, M.	1940.	*J. Gen. Physiol.,* xxiv, 15.
LA COUR, L. F.	1944.	*Proc. Roy. Soc. Edin.,* pt. 1, sect. B, lxii, 73.
LEACH, E. H.	1945.	This *Journal,* lvii, 149.
MAXIMOW, A. A.	1927.	*Arch. Path.,* iv, 557.
RASTELLI, M.	1943.	*La puntura sternale, Rome,* p. 53.
ROMEIS, B.	1932.	Taschenbuch der mikroscopisch Technik, 13th ed., *Munich* and *Berlin,* para. 255.

SANDERS, F. K. . . . . . . 1946. *Quart. J. Microscop. Sci.*, lxxxvii, 203.

STEDMAN, E. . . . . 1944. *Edinb. Med. J.*, li, 353.

    ,, ... 1945. *Biochem. J.*, xxxix (Proc. Biochem. Soc.), p. lviii.

THORELL, B. . . . . 1944. *Acta med. Scand.*, cxvii, 334.

TURNBULL, H. M. . . . . . . 1936. *In* The anæmias, by J. M. Vaughan, 2nd ed., *London*, p. 15.

VAN DEN BERGHE, L., AND HOFF- 1945. *C.R. Soc. biol. Paris.*, cxxxix, 324.
MAN, J.

WHITE, J. C., BAKER, J. R., AND 1946. This *Journal*, lviii, 155.
GRIFFIN, J. G.

Addendum

(Footnote to p. 227)

It has subsequently been found better to prepare the test solution by adding an equal volume of twice-isotonic veronal-acetate buffer at pH 6·85 to the heated aqueous solution of ribonuclease; this gives very complete removal of basophilic material in 1 hour at 37° C. The control tissue in isotonic buffer alone shows perfect preservation of basophilia, with subsequent brilliant staining by pyronin. Since carrying out this modification, which will be dealt with in greater detail in a future communication, the paper by F. K. Sanders (1946), in which a similar technique is used, has come to my notice.

THE INFLUENCE OF VIRULENCE ON THE IMMUNISING POTENCY FOR MICE OF *HÆMOPHILUS PERTUSSIS*, PHASE I

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The increasing confidence in *pertussis* immunisation, based on many favourable reports on its field application, was disturbed by the failure of the recent trials in Oxford to demonstrate any protection in children following the use of a phase I vaccine (McFarlan, Topley and Fisher, 1945). It is understood (McFarlan, 1946, personal communication) that it was not the intention of these workers to discredit *pertussis* vaccination as a practice, but merely to show that a vaccine produced by a reputable institution from phase I organisms was not *ipso facto* an effective prophylactic agent. Moreover, McFarlan and his colleagues in their paper suggested the possibility that the vaccine may have been at fault and that, although it was possible to criticise points in the planning or statistical analysis of some of the successful American trials, it was difficult to avoid the conclusion that vaccination conferred some protection.

Previous experiments (Gray, 1946) showed that significant variations may occur in the mouse virulence of a single strain of *H. pertussis*, determined by the intranasal route, within the present concept of phase I. Enhancement in virulence is influenced by decreasing physiological age of the cultures and is most evident after 24 hours' incubation. Furthermore, growth in the developing egg produces organisms which are more virulent than those of equivalent age on Bordet-Gengou medium.

The experiments here recorded represent an attempt to provide evidence that not all phase I vaccines possess equal immunising potency and that vaccines made from highly virulent young cultures are more suitable for vaccine production than the older cultures hitherto employed in animal and human vaccination.

Immunising potency is a purely relative character, dependent upon the animal species and the experimental conditions, and it is therefore unwise to attempt to relate directly the results of experiments in mice to the response in children. But I feel that my results offer some indication of the possible reason for the failure of the Oxford trials.

Origin and maintenance of H. pertussis

The original strain of *H. pertussis* (Gray), isolated directly from a cough plate and vacuum-dried in a large number of ampoules, was used throughout for preparing vaccines and for determining the degree of protection they afforded.

Preparation and standardisation of vaccines

For the initial experiments vaccines were prepared from the suspensions used in the original virulence tests, because it was desired to correlate virulence with immune response. For later work fresh vaccines were prepared from dried cultures of *H. pertussis*.

(*a*) *Bordet-Gengou* (*B-G*) *vaccines.* The physiological age of the cultures used for vaccine production was recorded as the time elapsing from subculturing from an ampoule recovery plate at 60 hours until the growth was harvested by washing off in 0·5 per cent. phenol-saline. On this basis a number of subculture plates sown simultaneously were harvested at appropriate intervals to provide 24-, 48- and 72-hour vaccines. The suspensions were standardised to 10,000 million organisms per ml. and stored in the cold until used. As before, the standard of reference was Wellcome (Brown's) no. 9 opacity tube, checked by dilution 1 : 4·5 to no. 2 tube. Where dilutions were necessary to give varying doses of vaccines these were made in phenol-saline immediately before administration.

(*b*) *Egg vaccines.* Six- or seven-day chick embryos were inoculated directly into the allantoic cavity with a 24-hour B-G suspension, harvested at the appropriate time and concentrated and standardised in the manner previously described. Merthiolate (1 : 10,000) replaced phenol as preservative in this series because of the action of phenol on egg protein.

Doses

In these experiments vaccines were administered subcutaneously or intranasally and doses were made considerably smaller than those employed by many workers, with the object of approximating more nearly to the dose for a child on a weight-for-weight basis. It was also found convenient in most cases to reduce the number of injections to a minimum and to allow only a short period for the establishment of immunity. This method is contrary to the practice in children but it was justifiable here, since the experiments were purely a comparison of the potency of different vaccines and it was not therefore desired to achieve complete protection in any group. Only in the final experiment was any attempt made to assess the value of decreasing doses of the same vaccine administered by different routes, as an indication of the possibility of reducing the relatively enormous dose of 90,000 million organisms now recommended for children (Sauer, 1935).

Experimental mice

White mice of the disease-free strain previously used were given the first dose of vaccine—sometimes the only dose—at 3 weeks of age and, after the appropriate rest period for each experiment, received a challenging dose of living organisms. Although these mice may be considered too immature to respond well to immunisation, this age was chosen to conform with previous virulence tests. Moreover, as stated above, the experiments were not designed to afford complete protection in any group of mice, thus showing the contrasts to greater advantage. Despite their immaturity, however, the immune response was quite well marked.

Method of infection and assessment of degree

Following the method of Burnet and Timmins (1937), the immunity of the mice was challenged by instilling a dose, ranging in different experiments from 25 million to 100 million organisms, intranasally under ether-chloroform anæsthesia. The diluent used was buffered B-G broth (Gray) and the test dose (0·05 ml.) was administered with a standard dropping pipette. Mice were discarded if they failed to inhale the dose evenly or did not recover satisfactorily from the anæsthetic. Confirmation of the cause of death was secured by autopsy and smear in each case.

Interpretation

The degree of protection afforded by different vaccines was assessed in terms of percentage survival over a period of three weeks, of daily mortality rate, of average survival times and, in some cases, of the degree of lung involvement shown by the survivors. For computing average survival times the practice was adopted, as previously, of recording all animals alive at the 21st day as having survived for only 22 days, although many would have survived indefinitely. The significance of observed differences in protection was determined by Fisher's exact χ^2 method of statistical analysis.

RESULTS

Reference to the previous results (Gray) will show that the most virulent form of *H. pertussis* phase I obtained was a 24-hour culture on B-G medium and that virulence declined very steeply as the period of incubation was extended to 48 hours or longer. Although egg cultures of equivalent age were found to be more virulent than B-G cultures, it was not practicable to harvest the eggs at less than 48 hours. Egg cultures of this age were not significantly less virulent than 24-hour B-G cultures, but as they are rather more difficult to prepare, the main emphasis in this paper has been placed on the young B-G cultures. Comparative trials of B-G and egg vaccines will be recorded later.

The influence of physiological age on the immunising potency of H. pertussis *phase I*

The vaccines used in this and in some of the following experiments were made by adding 0·5 per cent. phenol to the suspensions used in the original virulence tests. When necessary, fresh vaccines were prepared in the manner described above. A sample of Wellcome 72-hour vaccine was obtained for comparison with our own preparations. This was not from the same batch as that used by the Oxford workers, which had then exceeded the expiry date, but was prepared under identical conditions from the same cultures of *H. pertussis* and may therefore be considered comparable in potency.

The mouse-immunising dose (1500 million organisms) was injected subcutaneously and after a rest period of fifteen days a challenging dose of 100 million 24-hour organisms was administered intranasally under anæsthesia. Table I summarises the experiment.

Statistical comparison of the protection afforded by the different vaccines is expressed in terms of probability (P) which is interpreted as significant at the level 0·05 and highly significant at 0·01 or less. The probability of survival in the series tested, namely 24-hour, 48-hour, 72-hour, Wellcome vaccine and controls, is 86·3, 65·3, 40·7, 40 and 2·4 per cent. respectively. All the observed differences are

TABLE I

Comparison of 24-hour, 48-hour and 72-hour B-G vaccine with Wellcome 72-hour vaccine (Oxford trials 1945)

Series	Vaccine	Death (time in days)	Survival		Significance
			No.	Per cent.	
A	24-hour B-G	3, 3, 3, 3, 4, 4, 7, 9, 10, 20	63/73	86·3	v. 48 hr. P=0·008 v. combined 72 hr. P=0·001 v. controls P=0·001
B	48-hour B-G	2, 3, 3, 3, 3, 4, 5, 5, 6, 6, 6, 8, 8, 8, 8, 13, 14, 14, 17, 20, 20, 20, 20, 20, 20	47/72	65·3	v. combined 72 hr. P=0·009 v. controls P<0·01
C	72-hour B-G	2, 2, 2, 2, 2, 3, 3, 3, 3, 5, 5, 5, 5, 5, 7, 12	11/27	40·7	v. controls P<0·01
D	Wellcome (72-hour)	1, 2, 2, 2, 2, 3, 3, 3, 3, 4, 4, 5, 6, 6, 10, 10, 17	12/30	40·0	
E	Nil (Controls)	1, 2, 2, 3, 3, 3, 3, 3, 3, 4, 5, 5, 5, 5, 5, 5, 5, 8, 8, 8, 8, 8, 9, 11, 11, 11, 13, 13, 15, 15, 15, 15, 16, 16, 16, 16, 18, 18, 19, 20, 20	1/42	2·4	

highly significant and are in accordance with expectation if virulence, as judged according to physiological age, serves as a measure of immunising potency. The two 72-hour vaccines, our own and that of the Wellcome Laboratories, give statistically indistinguishable results, with protection of a low order, and for analysis it is convenient to combine these two series.

Fig. 1, in which percentage mortality is plotted against time, shows the relative efficacy of each series at any stage during the experiment.

Comparison of egg and B-G vaccines

This experiment examines further the relationship of virulence to immunising potency, comparing cultures of different physiological age, grown on B-G medium and in the developing egg, of which the virulence has already been determined (Gray). Table II summarises the findings of a representative experiment using these vaccines.

The results show, first, that the relationship between physiological age and virulence (Gray) is reflected in immunising potency, whether the culture medium is B-G or the developing egg, that is to say, the younger the culture, the greater the virulence and the immunising potency. Second, there is no significant difference between the
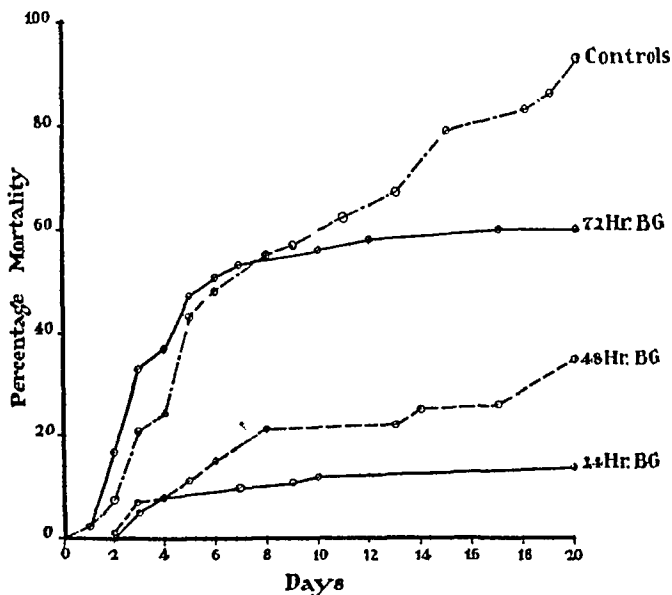
FIG. 1.—Mortality rate of 24-hr., 48-hr., and 72-hr. vaccines (combined figures).

immunising potency of the 24-hour B-G vaccine and the older (48-hour) egg vaccine, nor between the 48-hour B-G vaccine and the 4-day egg vaccine. By inference from these figures it is suggested that a 24-hour egg vaccine would be superior to a 24-hour B-G vaccine and statistical analysis of the trend of effectiveness with physiological age confirms this suggestion. Moreover it is probable,

other things being equal and assuming that a method could be devised to obtain an adequate yield from either B-G or egg cultures in less than the minimal times here recorded, further reduction in physiological age would give even more efficient vaccines.

TABLE II

Comparison of egg and B-G vaccines of varying physiological age

Series	Vaccine	Death (time in days)	Survival			Significance
			No.	Average time (days)	Per cent.	
A	24-hour B-G	9, 20	43/45	23·5	96	v.B, P=0·24 v.C, P<0·005 v.D, P<0·005
B	48-hour egg	11, 11, 20, 20, 20	38/43	22·2	89	v.C, P>0·05 v.D, P>0·05
C	48-hour B-G	5, 6, 8, 8, 8, 13, 17, 20, 20, 20, 20, 20, 20	31/44	21·1	70	v.D, P>0·05
D	4-day egg	2, 3, 3, 6, 6, 7, 7, 15, 17, 20	20/30	18·8	67	v.E, P<0·01
E	Nil (Controls)	1, 2, 2, 2, 2, 2, 2, 2, 2, 2, 3, 3, 3, 3, 5, 8, 8, 9, 9, 13, 13, 15, 15, 15, 18, 18, 19, 20, 20	1/30	8·6	3·3	...

Immunisation: single dose of 1500×10^6 organisms injected subcutaneously. Challenging dose after 15 days: 50×10^6 24-hour B-G organisms intranasally. Termination: at 23rd day. For averaging, survivors recorded as 24 days.

Interpretation of the results in terms of average survival times and of degree of lung involvement in animals dying or surviving, although confirming the above conclusions, failed to supply additional statistical evidence to that already obtained from percentage survival at the end of the experiment.

Comparison of vaccines by intranasal and subcutaneous administration

Intranasal administration of *pertussis* vaccines to mice as a method of assay was recommended by Dow (1940). Her results suggested that it was possible by this method to separate whole *pertussis* vaccines and soluble vaccines into two groups of high and low potency respectively. Within the group of whole vaccines, however, the products of different laboratories, administered intranasally in the same volume to mice, could not be separated despite very marked differences in their cell content, preservative and nitrogen content. It should be noted that Dow gave relatively large doses of vaccine to mice, ranging from 2250 million to 7750 million, representing, on a weight-for-weight basis, from 20 to 86 times the maximum recommended dose for children. Moreover a very large challenging dose—

2250 million 48-hour organisms—was employed and it is not improbable that these experimental conditions, compared with those employed here, would effectively mask any except dramatic differences in protective potency. Dow showed that a much greater protection was afforded, dose for dose, by the intranasal than by the intraperitoneal route, even under these severe experimental conditions.

North and Anderson (1942), reporting work carried out concurrently with that of Dow and examining some of her findings, confirmed the greater protective power of vaccines administered by the intranasal route. They suggested that the enhanced immunity is composed of (1) a general specific immunity due to circulating antibodies and (2) a local immunity in the respiratory tract, in which non-specific factors play a large part. These workers suggest, in view of the important influence exerted by non-specific factors on mouse immunity, that it would appear safer to use a method of assay in which these factors play no part.

This experiment examines the protective potency of 24-, 48- and 72-hour vaccines, given by the intranasal route, as compared (a) with each other and (b) with a control series receiving different doses of the 24-hour preparation given subcutaneously. The vaccine dose is small in the main comparison (300 million), in contrast to the doses used by Dow, and a relatively small challenging dose is given (50 million 24-hour organisms).

Table III summarises this experiment and fig. 2 plots the death-rate against time, showing the relative merits of each vaccine at any stage of the experiment.

The slightly longer rest period than that allowed previously was adopted because of North and Anderson's suggestion that, although the immunity following intranasal vaccination was not evident by the tenth day, it was well established by the thirty-eighth day.

The following statistical comparisons of percentage survival were made.

Twenty-four-hour vaccine intranasally. At the 300-million dose level (series A, table III) the 24-hour vaccine gives significantly greater protection than (B) the 48-hour vaccine, (C) the 72-hour vaccine in the same dose intranasally, (F) the 24-hour vaccine in the same dose subcutaneously or (G) the 24-hour vaccine at one-tenth of the dose intranasally. Its observed superiority over a fivefold increase in dose given subcutaneously almost reaches the level of significance. The general conclusion drawn is that the 24-hour vaccine is superior to the other two in equivalent doses and that its efficiency in mice is greater by the intranasal than by the subcutaneous route.

Comparing the relative efficiency of the two routes of immunisation in discriminating between the three vaccines, statistical analysis suggests that the intranasal method is more selective for antigenic differences. That is to say, whereas by the subcutaneous route the

24-hour *v.* 72-hour comparison shows a difference of 46 per cent., it is increased to 60 per cent. by the intranasal route.

TABLE III

Intranasal v. *subcutaneous inoculation of vaccines*

eries	Vaccine	Route	Dose of organisms	Death time in days	Survival			Significance
					No.	Average time in days	Per cent.	
A	24-hour	I/N	300×10^6	3	24/25	21·2	96	v.B, P = 0·009 v.C, P < 0·009 v.E, P = 0·10 v.F, P = 0·15 v.G, P = 0·001
B	48-hour	I/N	300×10^6	5, 6, 6, 6, 6, 7, 8, 9, 12, 20	15/25	16·6	60	v.C, P = 0·17
C	72-hour	I/N	300×10^6	4, 4, 4, 5, 5, 5, 5, 6, 6, 6, 6, 10, 12, 16, 17, 20	9/25	13·9	36	v.D, P = 0·03
D	Saline control	I/N	Nil	2, 4, 4, 4, 6, 7, 8, 9, 9, 10, 10, 11, 12, 12, 14, 16, 16, 16, 16, 17, 18, 20, 20	2/25	12·2	8	...
E	24-hour	S/C	1500×10^6	3, 4, 4, 4, 8, 12	19/25	18·1	76	v.F, P = 0·6
F	24-hour	S/C	300×10^6	3, 4, 4, 5, 5, 5, 9, 13, 16	16/25	16·6	64	v.D, P = 0·01
G	24-hour	I/N	30×10^6	1, 2, 9, 9, 10, 11, 12, 12, 13, 13, 15, 17	13/25	16·5	52	v.D, P < 0·01

Vaccines administered in 2 doses by either the subcutaneous (S/C) or intranasal (I/N) route, with a 7-day interval between doses. Rest period three weeks. Challenging dose, 50 million organisms of a 24-hour B-G culture intranasally. Mice were observed for three weeks after the challenging dose and, for determining average survival times, mice still living are recorded as having survived 22 days.

Other comparisons. With the number of mice employed (25) the observed difference between the 48-hour and 72-hour vaccines (24 per cent.) is not significant, but in view of the results of the previous experiments it is not considered necessary to repeat this comparison.

Comparing the probability of survival among mice receiving a 30-million intranasal dose of 24-hour vaccine (G) with those receiving ten times this dose of either 48-hour (B) or 72-hour vaccine (C) by the same route we find no significant difference. In other words the 24-hour vaccine is at least ten times as efficient as the other two at these dose levels.

DISCUSSION

There can be no question that obviously rough variants of *H. pertussis* (phases II, III or IV) are valueless as immunising agents. Moreover attempts to isolate from the smooth phase antigenic fractions capable of stimulating as effective an immunity as whole vaccines have not succeeded.

		Percentage		Mortality						
24Hr 300×10⁶ I/N		4		20	28	32	36			40
48Hr 300×10⁶ I/N			12	44		48	52		56	60	64
72Hr 300×10⁶ I/N		4	16	20	28	44	50	60	76	84	92
Controls										
24Hr 1500×10⁶ S/C			16		20		24				
24Hr 300×10⁶ S/C			12	24		28		32	36		
24Hr 30×10⁶ I/N		8			20	32	40	44	48		

Fig. 2.—Intranasal *versus* subcutaneous administration of vaccines.

Dealing with this problem on the basis of mouse experiments it is evident that vaccines prepared from the virulent form of *H. pertussis*, namely phase I, confer some degree of protection on mice. Previous experiments (Gray) showed, however, that the virulence of phase I *H. pertussis* is not constant but varies considerably under the influence of a number of factors. The most important of these yet detected are the physiological age of the culture and the medium used.

The experiments here recorded show that virulence of *H. pertussis* phase I is a useful index of protective potency. No matter whether the vaccine is introduced intranasally or subcutaneously, 24-hour vaccine is significantly superior to vaccines prepared from older cultures and the degree of protection afforded correlates well with predictions based on observed differences in virulence.

Neither endotoxin, capsule nor any other major antigen alone appears to play a significant part in determining virulence (Gray) or antigenicity. It appears that virulence and antigenicity depend on the presence of certain labile factors in the cell which are most pronounced in young cultures. That these factors are independent of the accepted phase I state of the organism is readily shown by the agglutinability of cultures of different physiological age with phase I antiserum. The most effective vaccine, therefore, is one which retains all these factors in a relatively unaltered state. Hence it appears that these observations offer some justification for an optimistic view on the effectiveness of active immunisation in children. The success of some workers (Macdonald and Macdonald, 1933; Sauer, 1933*a*, 1933*b*, 1935 and 1939; Sauer and Tucker, 1942; Kendrick and Eldering, 1936; Howell, 1938; Schermerhorn, 1938; Bell, 1941; Tucker, 1941; Daughtry-Denmark, 1942; Perkins *et al.*, 1942) may be due to their use of vaccines prepared from cultures of which the physiological age did not exceed 48 hours. Of those failing to achieve protection, at least one group of workers (McFarlan *et al.*) used a 72-hour vaccine.

Although one hesitates to relate results in mice to those in children, it is felt that some indication of the possible reason for the failure of the Oxford trials is to be found in the physiological age of the vaccine used. This reasoning cannot be invoked, however, to account for the failure of the trials by Siegel and Goldberger (1937) and Doull *et al.* (1939). Both these groups of workers used 48-hour vaccines. The only argument tenable here is that the 48-hour vaccine is relatively inefficient in protecting mice compared with the 24-hour preparation. Therefore, if a physiologically young vaccine' were used one might reasonably expect it to confer some protection.

Considering the results of table III one might reasonably expect to achieve much the same degree of protection as appears to be conferred on children by commercial 48-hour vaccines with approximately one-tenth of the recommended dose (90,000 million organisms) of

24-hour vaccine. Such a reduction in dosage would be of considerable importance in alleviating the discomfort associated with *pertussis* vaccination.

Local *versus* general immunity

It is not unlikely that the enhanced immunity conferred by intranasal over subcutaneous vaccination is in part non-specific, in the form of residual interstitial mononuclear aggregations, but it is possible that local specific factors also play a part. This suggestion raises once more the widely discussed question of whether a local as opposed to a general specific immunity may be stimulated by a particular immunological experience. If we accept the general hypothesis that humoral immunity arises solely or in great measure from the activity of the reticulo-endothelial cells, it would be expected that specific local immunity of a particular organ or tissue would depend on the presence of sessile R-E cells capable of antibody production. Such cells, once trained to produce a specific antibody, would give rise to a local anamnestic response with an immediate effective local concentration of antibodies. In support of this suggestion, Boyd (1943, p. 62, footnote) states "There is some indication that antibodies can be manufactured at least to some extent locally in almost any part of the body".

Regarding the protection afforded by egg vaccines compared with B-G vaccines of the same physiological age, it is considered that the developing egg provides more adequately the nutritional environment necessary to the optimal growth of *H. pertussis* outside the human body. Failure to carry investigations further on egg vaccines from cultures of less than 48 hours' incubation—which might reasonably be expected to be superior to 24-hour B-G vaccine—was due to technical difficulties in securing an adequate harvest from the egg in less than 48 hours. It is felt, however, that this method of cultivation offers interesting possibilities with nutritionally exacting organisms such as *H. pertussis*.*

Examining the trend of vaccine efficiency with decreasing physiological age, in both B-G and egg cultures, one finds it is probable that still further reduction of the physiological age—assuming that a method could be devised to obtain an adequate yield in this time— would give an even more efficient vaccine.

No opportunity has yet occurred to test the validity of the assumption that a 24-hour vaccine would prove a far more effective immunising agent for children than either the standard 48-hour vaccine, or the less commonly used 72-hour or older vaccines. However, on the basis of these results it is felt that such a trial is warranted.

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* In any development of egg-cultured vaccines for human use due recognition would be necessary of the not uncommon sensitivity, especially of children, to egg substances. Curphey (1947) records a fatal hæmorrhagic allergic reaction in a child following the injection of egg cultured influenza vaccine.

SUMMARY

1. The variations in virulence of phase I *H. pertussis* induced by altering the incubation time and the conditions of growth are reflected in the immunising potency for mice of the corresponding vaccines.

2. The intranasal route of vaccination confers a greater degree of protection than the subcutaneous route and, contrary to the results of other workers, appears to be suitable for demonstrating fine differences in the antigenicity of whole vaccines.

3. A possible explanation of the failure of the Oxford trials to protect children is offered, namely that the vaccine used was physiologically old and therefore of poor protective quality.

4. It is considered that the results of these experiments are sufficiently encouraging to justify a well-organised field trial using 24-hour vaccine under carefully controlled conditions.

REFERENCES

BELL, J. A. . . . . 1941. Publ. Hlth. Rep., *Washington*, lvi, 1535.
BOYD, W. C. . . . . . . 1943. Fundamentals of immunology, *New York*, p. 62 (footnote).
BURNET, F. M., AND TIMMINS, C. . 1937. *Brit. J. Exp. Path.*, xviii, 83.
CURPHEY, T. J. . . . . . 1947. *J. Amer. Med. Assoc.*, cxxxiii, 1062.
DAUGHTRY-DENMARK, L. . . . 1942. *Amer. J. Dis. Child.*, lxiii, 453.
DOULL, J. A., SHIBLEY, G. S., 1939. *Ibid.*, lviii, 691.
   HASKIN, G. E., BANCROFT, H.,
   McCLELLAND, J. E., AND HOEL-
   SCHER, H.
DOW, R. P. . . . . 1940. *Canad. Publ. Hlth. J.*, xxxi, 370.
GRAY, D. F. . . . . . . 1946. *Austral. J .Exp. Biol. and Med. Sci.*, xxiv, 301.
HOWELL, C. . . . . . . 1938. *Southern Med. J.*, xxxi, 1166.
KENDRICK, P., AND ELDERING, G. 1936. *Amer. J. Publ. Hlth.*, xxvi, 8.
MACDONALD, H., AND MACDONALD, 1933. *J. Inf. Dis.*, liii, 328.
   E. J.
McFARLAN, A. M., TOPLEY, E., 1945. *Brit. Med. J.*, ii, 205.
   AND FISHER, M.
NORTH, E. A., AND ANDERSON, G. 1942. *Med. J. Austral.*, ii, 228.
PERKINS, J. E., STEBBINS, E. L., 1942. *Amer. J. Publ. Hlth.*, xxxii, 63.
   SILVERMAN, H. F., LEMBCKE,
   P. A., AND BLUM, B. M.
SAUER, L. W. . . . . . . 1933*a*. *J. Amer. Med. Assoc.*, c, 239.
   ,, ... 1933*b*. *Ibid.*, ci, 1449.
   ,, ... 1935. *Amer. J. Publ. Hlth.*, xxv, 1226.
   ,, ... 1939. *J. Amer. Med. Assoc.*, cxii, 305.
SAUER, L. W., AND TUCKER, W. H. 1942. *Amer. J. Dis. Child.*, lxiii, 199.
SCHERMERHORN, L. J. . . . . 1938. *J. Pediatr.*, xiii, 279.
SIEGEL, M., AND GOLDBERGER, 1937. *J. Amer. Med. Assoc.*, cix, 1088.
   E. W.
TUCKER, W. H. . . . . . . 1941. *Illinois Hlth. Messenger*, xiii, 37.

615 . 778 (penicillin+streptomycin) : 576 . 856 . 7

THE SENSITIVITY OF ORGANISMS OF THE GENUS LEPTOSPIRA TO PENICILLIN AND STREPTOMYCIN

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Leptospira icterohæmorrhagiæ was at first reported to be insensitive to penicillin *in vitro* (Abraham *et al.*, 1941) but later workers asserted that strains in their possession showed considerable sensitivity, both *in vitro* and *in vivo* (Alston and Broom, 1944 ; Borg Petersen and Schmidt, 1945 ; Larson and Griffiths, 1945). It appeared probable therefore that this group of organisms might show some strain variation in susceptibility to penicillin such as has been found with other micro-organisms. Observations regarding leptospiræ other than *L. icterohæmorrhagiæ* have been scanty. The effect of streptomycin has been investigated fairly extensively *in vivo* and its value relative to penicillin will be discussed later in this paper.

We thought it worth while to test *in vitro* the susceptibility of all the 29 organisms of the genus Leptospira in our possession, including six strains of *L. icterohæmorrhagiæ* from different sources.

The investigations comprise two experiments. In the first, a study was made of the general antibiotic effect *in vitro* of the two substances, penicillin and streptomycin, in relatively high concentration upon the whole range of species of Leptospira in this laboratory. In the second, in view of the desirability of correlating the effect of penicillin on the cultivated organisms with the blood concentrations at present practicable in clinical work, the common pathogens of the genus Leptospira were tested against higher dilutions in order to determine the approximate end-point of their inhibition by penicillin.

METHOD

The organisms were grown in a medium of 12 per cent. rabbit serum in water double distilled from glass. All the serum required for the investigation was taken from rabbits whose sera had previously been shown to support the growth of the leptospiræ under investigation—a necessary precaution, because some rabbit sera do not support the growth of leptospiræ and a few contain

antibodies which agglutinate *L. icterohæmorrhagiæ*. One batch of the serum-water medium was prepared and divided into five equal volumes, one of which was left as a control. To the remaining four either penicillin or streptomycin was added according to the following scheme :—

(1) 1 unit penicillin per ml.
(2) 10 units   ,,   ,, ,,
(3) 5 units streptomycin per ml.
(4) 50 units   ,,   ,, ,,

The four separate solutions and the control were then put into tubes measuring 25×2 cm., each tube receiving 5 ml. of the medium. The species of Leptospira to be tested were separately inoculated into one tube of each of the above groups, the inoculum being 1 ml. of the stock culture. Since the stock cultures were of approximately equal density the number of leptospiræ delivered in each case was about the same. All the tubes were incubated at 28° C. and the cultures examined macroscopically and microscopically after 2 and 3 days.

Whereas streptomycin is stable, penicillin deteriorates on incubation; therefore, to check the loss of activity over the period of investigation, all tubes to which penicillin had been added were tested for potency at the end of the experiment by the cylinder plate method (Heatley, 1944) against the Oxford H strain of *Staphylococcus aureus*. The samples from the tubes to which 10 units had been added were diluted 10 times before assay. Upon each plate a standard of 1·0, 0·75 or 0·5 unit per ml. was placed in addition to 6 or 7 samples. A graph constructed from the size of the standard rings showed that after 3 days' incubation approximately $\frac{1}{4}$-$\frac{1}{3}$ of the penicillin had been lost, whether the leptospiræ had grown or not.

Because *L. icterohæmorrhagiæ* is by far the most important pathogenic organism of this genus, six strains of diverse origin were investigated (table I).

TABLE I

Strains of Leptospira icterohæmorrhagiæ *investigated*

Strains	Source
L. icterohæmorrhagiæ A. R.G.A.	Original *icterohæmorrhagiæ* strain isolated from a guinea-pig infected with human material from a case of Weil's disease on the Western Front in 1915 (Uhlenhuth and Fromme, 1916)
L. icterohæmorrhagiæ AB. M.20 .	Borg Petersen, 1938
L. icterohæmorrhagiæ (Cockburn)	Highly virulent for guinea-pigs ; maintained for 2 years by passage (Wylie, 1946a)
L. icterohæmorrhagiæ (Wijnberg)	National Collection of Type Cultures, Lister Institute, London
L. icterohæmorrhagiæ (Vady) .	Ditto, no. 4633
L. icterohæmorrhagiæ (Buckland)	Weakly virulent for guinea-pigs

The remaining species, of European and oriental origin, were obtained by the courtesy of Dr C. Borg Petersen of the State Serum Institute, Copenhagen. The source has been given of the less common resistant species.

Results

Table II summarises the results at 3 days. The growth was recorded from the density of the suspension of living leptospiræ as assessed by dark-ground microscopy.

Table II

Inhibition of 29 strains of Leptospira by penicillin and streptomycin

			3 days			
		Control	Penicillin		Streptomycin	
			1 unit	10 units	5 units	50 units
L. icterohæmorrhagiæ A	.	>10	—	—	1	—
" " AB	.	>10	—	—	—	—
" " Buckland	.	>10	1-2	<1	5	5
" " Vady	.	>10	—	—	—	—
" " Cockburn	.	>10	—	—	1	—
" " Wijnberg	.	>10	<1	—	1	—
L. grippotyphosa	.	>10	—	...	<1	—
L. canicola	.	>10	—	—	—	—
L. sejroe	.	>10	—	—	—	—
L. djasiman	.	>10	—	—	—	—
L. bataviæ	.	>10	—	—	—	—
L. pomona	.	5	—	—	—	—
L. australis a (Cotter, 1935, quoted by Clayton and Derrick, 1937)		>10	—	—	2	2
L. poi	.	5	—	—	—	—
L. sari	.	>10	—	—	—	—
L. pyrogenes	.	>10	<1	—	—	—
L. hebdomadis	.	5	—	—	—	—
L. hc (Kouwenaar and Wolff, 1930)	.	5	<1	—	—	—
L. 3705 (Sumatran strain; Wolff and de Graaf, 1939, p. 2470)		>10	—	—	2	<1
L. autumnalis	.	>10	—	—	—	—
L. rachmat (Sumatran strain; Baerman)*		>10	—	—	2	<1
L. sentot	.	>10	—	—	—	—
L. australis b	.	>10	—	—	—	—
L. samarang (Javan strain; Sardjito and Mochtar, 1939)		>10	2	2	3	3
L. naam (Sumatran strain; Wolff and de Graaf, 1939)		>10	<1	—	—	—
L. benjamin	.	>10	—	—	—	—
L. javanica	.	>10	—	—	—	—
L. 90 c	.	>10	—	—	—	—
L. andaman a	.	>10	—	—	—	—

Streptomycin, 1 unit = 1 γ.

Penicillin, 1 Oxford unit = 0·6 γ approx.

The source is given of the less well known strains exhibiting resistance to the antibiotics.

The figures in columns 2-6 are the number of living leptospiræ per $\frac{1}{72}$ in. dark-ground field.

— = no leptospiræ, or dead leptospiræ only seen.

* Received from Borg Petersen.

None of the tubes with penicillin or streptomycin showed a richness of growth approaching that of the controls. In the majority, growth was materially diminished by the addition of penicillin or streptomycin

and, with few exceptions, penicillin was effective at a lower concentration than streptomycin.

Levels of 1 or even 10 units per ml. of penicillin can be maintained in the serum, but the dosage required for this is much greater than that now in common use (Lourie *et al.*, 1945). Thus it was thought advisable to determine on the commoner pathogenic leptospiræ the minimum concentration of penicillin which would totally inhibit their growth. For this test, the five relatively susceptible strains of *L. icterohæmorrhagiæ* were chosen, R.G.A., AB., "Cockburn", "Vady" and "Wijnberg", with the addition of *L. grippotyphosa*, *L. canicola* and *L. bataviæ*. Each strain was inoculated into 5 ml. of the same medium as before, with penicillin in concentrations of 0·025, 0·05, 0·1, 0·25 and 0·5 u/ml. A control tube without penicillin was inoculated with each strain and the test set up in quadruplicate. The inoculum was equal in each case—0·5 ml. of a 4-day growth of the stock culture.

From experience gained in the first investigation the inoculated tubes were incubated for 3 days at 28° C. before examination. Each tube was then examined microscopically and growth estimated by the number of actively motile leptospiræ seen in each 1/12th inch dark-ground field.

As before, it was thought advisable to determine to what extent the penicillin added at the start of the experiment had deteriorated after 3 days. The two higher concentrations, 0·5 and 0·25 units per ml., were estimated by the cylinder plate method of Heatley, but since a ring is not produced by a concentration less than 0·2 unit per ml., this method could not be used with the lower concentrations. In estimating the loss of penicillin in dilutions 0·025, 0·05 and 0·1 u/ml., resort was therefore made to the slide assay method of Garrod and Heatley (1944-45), with a drop from a Pasteur pipette as the unit of measurement. On each slide 4 tests were mounted, along with a control, *i.e.* a drop of medium from the control tube containing leptospiræ and a standard cell consisting of the control diluted with an equal quantity of a penicillin solution containing 0·04 unit per ml. (final dilution of 0·02 u/ml.). All these were then inoculated with a wire from a 1 : 1000 dilution of a 24-hour culture of the Oxford H strain of *Staphylococcus aureus*. This minute amount of the staphylococcal inoculum, it was deemed, would not significantly alter the quantity. The tubes to which the dilutions of penicillin had been added at the commencement of the experiment were similarly inoculated on the slide. The results of these tests showed that in the control without penicillin upwards of fifty colonies of *Staph. aureus* had grown after 24 hours' incubation at 37° C. With very few exceptions growth was entirely absent from the standard cell (final dilution 0·02 u/ml.) and from any to which penicillin in dilutions of 0·025, 0·05 or 0·1 u/ml. had been added before inoculation with leptospiræ.

Our findings may be summarised as follows —

Control *Staph aureus* without penicillin	. >50 colonies
Standard cell, 0 02 u/ml penicillin	nil (4 exceptions)
0 1 u/ml penicillin added 3 days previously	nil
0 05 „ „ „ „ „	nil
0 025 „ „ „ „ „	nil (3 exceptions)

In the 3 cases out of 15, and in the 4 exceptions where growth had occurred in the standard, all showed fewer than 10 colonies From this it is concluded that in the test tubes the penicillin had not deteriorated significantly below a concentration of 0 02 unit per ml

Where the growth of leptospiræ had been inhibited the tubes were left for three further days at 28° C, but the organisms did not recover and further subculture did not enable them to pick up Borg Petersen and Schmidt, however, found that after 28 days leptospiræ reappeared in dilutions of penicillin below 0 3 unit

During this research it was observed that the size of the inoculum, so far as this could be estimated, appeared to influence the growth of leptospiræ in media to which either penicillin or streptomycin had been added The larger the inoculum the more readily the organisms grew A large carry-over is necessary to ensure that the organisms will pick up in subculture Thus it is difficult to say from this observation how far the efficiency of the added inhibiting substance is influenced by inoculum size Nevertheless, the larger inocula used in this series may explain why the concentration of penicillin found necessary for complete inhibition of growth is slightly greater than that found by Borg Petersen and Schmidt

Table III shows the end point of the inhibition achieved by penicillin in the in-vitro cultures of the leptospiræ studied in this experiment

TABLE III

Inhibition of the commoner pathogenic leptospiræ by penicillin

Organism	Concentration of penicillin in units per ml				
	0 025	0 05	0 1	0 25	0 5
L icterohæmorrhagiæ R G A	Partial	Subtotal	Total		
„ AB	Partial	Total			
„ Cockburn	Subtotal	Subtotal	Total		
„ Wijnberg	Nil	Partial	Partial	Subtotal	Subtotal
„ Vady	Partial	Subtotal	Total		
L grippotyphosa	Nil	Nil	Partial	Total	
L canicola	Nil	Partial	Partial	Subtotal	Total
L bataviæ	Partial	Subtotal	Total		

DISCUSSION

Of the strains of *L icterohæmorrhagiæ* tested only " Buckland " was resistant to the higher concentrations of penicillin and streptomycin, while the classical R G A strain partially resisted the action

of 5 units per ml. of streptomycin. Unfortunately the strain "Jackson", originally reported resistant to penicillin (Abraham *et al.*), has died out and its degree of insusceptibility cannot be ascertained. The avirulent strain "Wijnberg" was not wholly inhibited by a penicillin concentration of 1 unit per ml. It would appear therefore that there are strain variations within this species of Leptospira which would account for the conflicting results so far reported.

The only other extensive investigation into the susceptibility of leptospiræ to penicillin is that of Borg Petersen and Schmidt, whose results, if their Danish units are converted into international units, are very similar to those here reported.

Of the remaining species investigated, the commoner European pathogens *L. canicola* (Walch-Sorgdrager, 1939) and *L. grippotyphosa* (Prausnitz and Lubinski, 1926; Buckland and Stuart, 1945) are susceptible to penicillin *in vitro* in concentrations below 1 unit per ml. *L. grippotyphosa* grew very sparsely in 5 units per ml. of streptomycin.

Of the strains investigated in the second experiment all were susceptible to concentrations below 1 unit per ml. except *L. ictero-hœmorrhagiœ* Wijnberg, which is avirulent.

Penicillin and streptomycin both severely retard the growth of all leptospiræ, but a greater number appear relatively less susceptible to streptomycin than to penicillin. This is in conformity with the experience of Waksman and Schatz (1945), who found penicillin more effective than streptomycin *in vivo*.

Value of penicillin and streptomycin in vivo

It is not justifiable to draw unqualified deductions from the results obtained *in vitro* concerning the probable efficiency of these antibiotics *in vivo*, but Larson and Griffiths and Borg Petersen and Schmidt found penicillin therapeutically effective against their strains of *L. icterohœmorrhagiœ*, provided that adequate concentrations could be established in the blood very early in the infection and thereafter maintained.

The only strain investigated extensively in this laboratory for virulence to the guinea-pig was the "Cockburn" strain of *L. ictero-hœmorrhagiœ*, with which it was found that unless penicillin (500 units three times a day) was administered within 24 hours of infection the animals died. These deaths may be explained by the observation that very early in the experimental infection profound changes occur in the renal circulation which may render the leptospiræ already present in that organ inaccessible to the circulating antibiotics (Wylie, 1946*b*). This might partially explain also the findings of Borg Petersen and Schmidt, who found leptospiræ in the kidneys of guinea-pigs which had been treated with penicillin 3 months previously. Guinea-pigs do not tolerate such massive doses of penicillin as might theoretic-

ally have achieved a sufficient concentration to kill the more resistant organisms (Hamre *et al.*, 1943 ; Borg Petersen and Schmidt, 1945).

Heilman (1945) found streptomycin effective in animal protection experiments. Waksman and Schatz investigated the effect of streptomycin and penicillin upon hamsters injected with *L. icterohæmorrhagiæ* and reported that both antibiotics were capable of preventing death, although penicillin appeared to be the better in that less was required for protection.

Reports on the beneficial effect of penicillin administration in cases of human Weil's disease have been guarded, as relatively few cases have been treated. Clinically the tendency is for the patients to recover spontaneously, and there are often periods of quiescence followed by relapse ; a very large series of cases would therefore be necessary for a conclusive demonstration of the value of penicillin therapy. In human infections with a sensitive strain it is reasonable to expect a lower relapse rate after continuous penicillin administration and this has in fact been reported by Bulmer (1945). Reports do not appear to be available as to the value of streptomycin in human Weil's disease.

Whether or not the leptospiræ grew in the dilution of penicillin tested, no significant difference was observed in the size of the rings produced. It seems likely, therefore, that none of the strains produced penicillinase under the conditions described.

SUMMARY

1. Twenty-nine strains of leptospiræ were tested *in vitro* against penicillin and streptomycin.

2. In the concentrations tested, both antibiotics partially or completely inhibited all the strains ; there was considerable variation in susceptibility between species, but most of the common pathogens were completely inhibited by 0·5 unit per ml. or less.

3. Strain variation in resistance was shown within the species *L. icterohæmorrhagiæ.*

4. On the whole, on a weight basis, penicillin was more inhibitory than streptomycin.

5. None of the organisms tested produced penicillinase.

REFERENCES

ABRAHAM, E. P., CHAIN, E. 1941. *Lancet,* ii, 177.
    FLETCHER, C. M., FLOREY,
    H. W., GARDNER, A. D.,
    HEATLEY, N. G., AND JENNINGS,
    M. A.

of 5 units per ml. of streptomycin. Unfortunately the strain " Jackson ", originally reported resistant to penicillin (Abraham *et al.*), has died out and its degree of insusceptibility cannot be ascertained. The avirulent strain " Wijnberg " was not wholly inhibited by a penicillin concentration of 1 unit per ml. It would appear therefore that there are strain variations within this species of Leptospira which would account for the conflicting results so far reported.

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SOME CHARACTERS OF COAGULATION NECROSIS DUE TO MUSTARD GAS

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(PLATE XXIX)

DURING the course of work on the action of mustard gas on the rabbit's eye (Mann and Pullinger, 1941-42) it became necessary to be able to classify this action in terms of general pathology. The provisional classification of the skin lesion as a blister or a burn was too wide to do more than relate it on the one hand to friction, ultra-violet light and many other vesicant agents or, on the other, to heat, caustic action and a variety of chemical burns. The long accounts by Warthin and Weller (1918-19) of experimental and post-mortem observations on animal and human lesions do not enable one to classify them except as necroses, nor do they help one to account for the fact that the corneal lesion in the substantia propria does not ulcerate out unless complicated by protracted vascularisation and lipoid degeneration. Dean and Swann (1924) observed slow progressive necrosis which extended into the deeper layers of the dermis of rabbits. The progressive nature of the lesion was emphasised by contrast with heat burns, in which the whole damage was found to be immediate. Heat burns did not progress nor was their course affected by subsequent degenerative changes. These authors ascribed the greater delay, by one week, in the healing of a mustard gas lesion to degeneration of blood vessels and to acute inflammation which were found for long periods after the application of a solution of 2 per cent. liquid mustard gas in alcohol. It is possible that this solvent modified the course of the lesion. In experiments described below using liquid mustard gas alone, vascular exudates and extravasation of red blood corpuscles occurred constantly but vascular degeneration was not a feature of the early stages of the process. The tissue was destroyed by coagulation necrosis ; a blood and lymph circulation existed within the lesion for several days while necrosis was extending ; the circulation was then arrested suddenly about the sixth to the ninth day and the dead mass was separated and lifted out like a sequestrum, apparently by the action of polymorphonuclear leucocytes in a dense marginal zone. The separation of a slough or sequestrum in this way was seen

also by Dean and Swann, as was the beginning of epithelial regeneration beneath the slough before its complete separation. The slow progress of the lesion seems to be due to delay in dying and being cast off rather than to delay in healing. In this devitalised state it is a suitable medium for bacterial growth. The actual cause of the separation of the slough is probably some other factor acting alone or together with the polymorphs, for these are present in the corneal lesion (Pullinger and Mann, 1943), yet the substantia propria does not slough out.

Chemical observations on the reactions *in vitro* of liquid mustard gas and collagen by Pirie (1947) and *in vivo* of liquid mustard gas and skin by Berenblum (1940) throw considerable light on the nature of the coagulation necrosis and may explain its peculiar features. These are referred to in the discussion.

Methods

The local action of mustard gas was examined on mouse skin and ears by techniques previously described (Pullinger and Florey, 1937 ; Pullinger, 1940). A very small drop of the liquid was delivered from a capillary pipette on to an area of skin at the back of the neck from which the hair had been clipped. At frequent intervals from 12 hours to 16 days afterwards two or three mice of the series were killed and the skin dissected off and fixed in Zenker's fluid. Unfixed frozen sections were also examined at earlier stages. Mouse ears similarly treated with liquid mustard gas coloured with sudan black (fig. 1) were examined in the living state with a binocular microscope from the moment of application and a parallel series by microscopy after fixation. Lymphatic injections were made with india ink as hydrokollag was not obtainable.' A tendency to leakage of india ink exists independently of any lesion and the leakages shown in the illustrations are probably due to this tendency alone. Permanent preparations were made by fixing the ears after injection in 10 per cent. formol-saline, dehydrating and clearing in glycerine and mounting in glycerine jelly.

Results

The time of onset of hyperæmia and œdema seen in the living ears varied considerably. Œdema was just apparent but not considerable in 2-3 hours and by then a few arteries and veins had become more conspicuous. By 12-15 hours the ears were swollen with œdema fluid. By this time, also, epidermal nuclei had become visible in unfixed frozen sections, indicating that these cells were now dead. By then, also, arteries and veins were engorged, while œdema extended beyond the treated region, as revealed by staining of the sebaceous glands with sudan black, where the liquid mustard gas had been applied (fig. 1). By the second day the whole ear was much swollen with fluid but it was possible to see blood still flowing through the treated region, which was pale compared with the rest of the ear. A few small-calibre vessels were visible in this pale zone and the blood flow through them could be interrupted by light pressure from a glass rod. When the rod was lifted the flow continued again. If the whole

pale region was gently stroked numerous small vessels filled with blood, revealing a larger potential circulation Pallor was due not to occlusion of all the vessels but to contrast with surrounding hyper-æmia and extravasation of red blood corpuscles Lymphatic channels in the lesion were also patent, as shown by micro injection with india ink (figs 2 and 3) These appearances remained almost unchanged for about 6 9 days, a time which varied from mouse to mouse Occlusion of all vessels within the lesion then became apparent for the first time (fig 4)

Meanwhile the fixed microscopic sections of skin and ears showed that a gradually extending and finally massive coagulation necrosis had already become recognisable by the second or third day The tissue was swollen , the shape of the epithelial cells, nuclei and connective tissue structures was preserved, though the staining reaction was defective in the cytoplasm and absent from the nuclei The contrast in staining capacity between this dead epithelium and epithelium stimulated to hyperplasia at the edge of the lesion was striking Previous to this, signs of cell death were scattered , there was gross swelling and œdema, a large fibrinoid coagulum lay beneath the panniculus carnosus equal in thickness to skin and subcutaneous tissue, and though actual fibrin could never be demonstrated, there was slight increase in leucocytes and macrophages, while here and there the epidermis was separated from the dermis Vessels containing blood corpuscles were patent, as were also the lymphatics Extravasated blood corpuscles were seen

After the 6th to the 9th day the dead tissue shrank *in situ*, became glass like and transparent in the ears and, in sections, retained only the outlines of the constituent parts Collagen became a poorly stainable homogeneous mass Hyaline thrombi were seen in some vessels An increasing number of polymorphs appeared in all specimens and bacterial colonies in many Separation of this dead and now bloodless tissue was gradually effected, apparently by the action in whole or in part of masses of polymorphonuclear leucocytes and by tongues of regenerating epithelium which dipped down from the hyperplastic edge between the dead and viable parts In mouse ears examined six months after application, the scar was avascular and devoid of lymphatics

The maintenance of a blood circulation after the appearance of coagulation necrosis rules out death by vascular occlusion and infarction Occlusion occurred 4 7 days later

The presence of a circulation through the œdematous tissue shows that the œdema fluid is attracted to and held in the lesion by an active osmotic force greater than that inside the vessels It is not a passive œdema due to lack of open channels for drainage A similar active force probably accounts for the obstinate corneal œdema

Discussion

According to chemical experiments on the interaction *in vitro* of liquid mustard gas and collagen an insoluble compound is formed which is not digested by proteolytic enzymes (Pirie). Berenblum found that liquid mustard gas gives a precipitate when added to cold water extracts of skin. This precipitate has a relatively high phosphorus content, suggesting that it contains nucleoprotein. The union of mustard gas with nucleoproteins was confirmed, using thymonucleoproteins.

The distinguishing pathological feature of coagulation necrosis is failure of the dead tissue to be absorbed or liquefied. It retains meanwhile the outline of the living minute structures, though not the same avidity, after fixation, for stains. In particular nucleo-chromatin fails to be stained, though the site and shape of the former nuclei and nucleoli may remain visible for a long time. It seems likely that this form of coagulation necrosis in contrast with other kinds of necrosis is due to relative insolubility of the dead product of tissue and poison. The poison may be laboratory made or produced by bacteria *in situ* (for example, diphtheria toxin), and it may have many other actions in the tissues unrelated to necrosis, especially those causing hyperæmia, œdema and exudates of cells and plasma. Agents of coagulation necrosis differ from good fixatives—for example mercury salts, formalin or picric acid, which also form insoluble protein conjugates—in that these permit subsequent vivid staining of cytoplasm and chromatin. The nuclear change in coagulation necrosis is usually described as a lysis. Nuclear chromatin, though no longer capable of being stained, may nevertheless be present in a new and insoluble compound. The fact that its site and shape and those of the nucleoli remain visible in the mustard gas lesion, though uncoloured, supports this view, as does the indirect evidence provided by Berenblum.

Soluble conjugates of mustard gas and serum proteins have been obtained by Berenblum and Wormall (1939). They were antigenic and gave rise to precipitins and complement-fixing antibodies on injection into horses, rabbits and guinea-pigs. Two other chemicals, benzoylchloride and o-chlorbenzylchloride, which are known to combine with proteins and which give rise to skin sensitivity after the manner of an antigen (Medveczky and Uhrovits, 1931; Landsteiner and Jacobs, 1936), were found by the same technique as was used for liquid mustard gas to cause coagulation necrosis, though not to the same depth in the skin. There is evidence therefore that combination of all these poisons with some of the tissue proteins results in coagulation necrosis and that this product is relatively insoluble. Soluble compounds also are formed with the same chemicals and other proteins, for example, serum proteins. It may be that these are responsible for the systemic sensitisation which is a feature of all of them. If however any part of the internal tissue is destroyed by coagulation

NECROSIS DUE TO MUSTARD CAS

FIG 1 —Portion of a mouse ear Distribution of the mustard gas is shown by sebaceous glands (A) coloured with sudan black ×10

FIG 2 —Lymphatics of another mouse ear injected with india ink 24 hours after application of mustard gas Coloured sebaceous glands are still visible and also some blood vessels (B) ×10

FIG 3 —Lymphatics of a mouse ear injected with india ink 4 days after application of mustard gas Sebaceous glands are no longer visible Several arteries and veins are patent and visible in the treated region (C) around ×10

FIG 4 —Lymphatics of a mouse ear injected 7 days after the application of mustard gas Lymphatics and blood vessels in the treated region (D) are occluded ×10

necrosis or any part near the surface fails to be cast off, it may serve as a long-continuing, very slowly soluble source of antigen. Absorption of the mustard collagen, prepared *in vitro*, does eventually take place when introduced under the skin of living animals (Pirie).

The *similarity in the appearance and behaviour of coagulation necrosis due to poisons and infarction due to sudden vascular occlusion has always been a puzzle*, because the two causes have apparently nothing in common. The observations here recorded do not provide any suggestion as to how tissues might be rendered insoluble through sudden cessation of their blood supply, yet appear to be of sufficient interest to be put on record and may stimulate the co-operation of biochemists in solving pathological problems which certainly have a chemical basis.

Summary

1. The local skin lesion caused by liquid mustard gas in mice is coagulation necrosis. It is accompanied by large vascular exudates.

2. Blood circulates through the lesion for a few days after necrosis has occurred and patent lymphatics traverse it.

3. Vascular occlusion occurs after 6-9 days. The dead tissue is then cast off as a slough.

4. The slow progress of the lesion is due to delay in dying and in being cast off. Marginal epithelial healing begins before final separation of the slough.

5. Collagen and nucleoproteins have been found by Pirie and by Berenblum respectively to combine with liquid mustard gas to form insoluble compounds.

6. It is suggested that the peculiar property of coagulation necrosis is failure to liquefy or be absorbed and that this failure is due to insolubility of the dead product of tissue and chemical compound.

REFERENCES

BERENBLUM, I. . . . . . . 1940. Ministry of Supply Reports.
BERENBLUM, I., AND WORMALL, A. 1939. *Biochem. J.*, xxxiii, 75.
DEAN, H. R., AND SWANN, M. B. R. 1924. Ministry of Supply Reports.
LANDSTEINER, K., AND JACOBS, J. 1936. *J. Exp. Med.*, lxiv, 625.
MANN, I., AND PULLINGER, B. D. 1941-42. *Proc. Roy. Soc. Med.*, xxxv, 229.
MEDVECZKY, A., AND UHROVITS, A. 1931. *Z. Immunitätsforsch.*, lxxii, 256.
PIRIE, A. . . . . 1947. *Biochem. J.*, xli, 185.
PULLINGER, B. D. . . . . . 1940. This *Journal*, l, 463.
PULLINGER, B. D., AND FLOREY, H. W. 1937. This *Journal*, xlv, 157.
PULLINGER, B. D., AND MANN, I. 1943. This *Journal*, lv, 151.
WARTHIN, A. S., AND WELLER, C. V. 1918-19. *J. Lab. & Clin. Med.*, iv, 229.

ADAPTATIONS OF THE MACFARLANE LECITHO-VITELLIN TEST *

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DETERMINATION of the toxigenicity of strains of clostridia or of the antitoxin titre of a serum by the injection of animals is a cumbersome, expensive, and time-consuming process, necessitating a large number of animals to discount the variables. While animal toxicity and protection is the direct and accurate approach and the standard method, a simple, inexpensive technique for a presumptive test should greatly facilitate investigation in fields in which toxigenicity of the clostridia is a problem, permitting greater numbers of determinations. Reports in the literature of the reaction of the alpha toxin of *Cl. welchii* on lipoprotein substrates have already suggested a basis for the development of such a simplified technique.

Seiffert (1939) noted that the toxin of *Cl. welchii* could produce an opalescence in normal human serum. He was unable to elicit this reaction with other toxins or with *Cl. welchii* toxoid and felt that it was not inhibited by specific antitoxin. Independently Nagler (1939) noted this same phenomenon, found that it was common to the toxins of all of Wilsdon's four types of *Cl. welchii*, and demonstrated that it could be prevented by specific antitoxin. Using this reaction he was able to titrate both unknown *Cl. welchii* antitoxin and un-unknown *Cl. welchii* toxin independently and found that his results compared well with those obtained by intravenous injection of toxin antitoxin mixtures. He also demonstrated that *Cl. œdematiens, Cl. septique, Cl. chauvœi, Cl. histo lyticum, Cl. sporogenes, Cl. tertium, Cl. tetani* and *Cl. botulinum* did not produce this opacity in serum.

Macfarlane, Oakley and Anderson (1941) were convinced that this reaction was produced only by the alpha toxin of *Cl. welchii* and showed that it could be prevented by any *Cl. welchii* antitoxin which contained sufficient alpha antitoxin. They also believed that the opalescence was due to free fat and that the fat was split from lipoproteins by the alpha toxin through an enzymatic reaction in the presence of ionized calcium. They compared the reactions of various mixtures of toxin antitoxin, using red cell hæmolysis, opalescence of human serum, and opalescence of a crude lecitho vitellin mixture prepared from egg yolk as end points, and found that the results ran parallel. Estimation of the minimal effective dose of alpha toxin against the same indicators showed that the lecitho vitellin mixture was the most sensitive.

Macfarlane and Knight (1941) proved conclusively that this phenomenon was governed by the dynamics of enzymatic reactions, and elaborated on the

factors which facilitated and inhibited its demonstration. They also showed that alpha toxin contains a lecithinase. Weed and his collaborators (Weed, Forney and Carter, 1942 ; Weed, Minton and Carter, 1942 ; Weed and Minton, 1942-43) were sceptical of the specificity and value of the reaction and presented evidence to substantiate their impressions. But close perusal of their data suggests that in some cases they were not observing the true reaction and that they were introducing extraneous factors which are known to inhibit or elicit the reaction. Also, these conclusions are in direct opposition to those arrived at by Crook (1942), Oakley and Warrack (1941), Hayward (1943), Lyons (1942) and van Heyningen (1941). Hayward, in adapting the Nagler reaction to a rapid method of identifying *Cl. welchii*, confirmed the value of the test, showed that some of the *sporogenes-sordelli-bifermentans* group produced a lecithinase, and provided evidence that while some non-toxigenic strains of *Cl. welchii* gave positive reactions, no toxigenic strain gave a negative reaction. van Heyningen presented data to confirm the specificity of the reaction and developed rather complicated, delicate test procedures for the accurate titration of alpha toxins and alpha antibodies.

Granted that the Nagler human-serum reaction and the lecitho-vitellin reaction of R. G. Macfarlane are due to the action of a lecithinase on a lipoprotein substrate, and that the alpha toxin of *Cl. welchii* contains a lecithinase, it should be possible to set up simple tests with proper controls demonstrating the presence of, and roughly estimating, alpha toxin and alpha antitoxin.

In this laboratory we have found a modification of the Macfarlane lecitho-vitellin reaction extremely useful for ascertaining the toxicity of strains of *Cl. welchii* and for estimating the amount of *Cl. welchii* alpha antibodies in sera.

Methods

I. Preparation of the lecitho-vitellin solution (Macfarlane et al., 1941). The yolk of one egg is shaken in a flask with glass beads and 250 c.c. sterile physiological saline until homogeneous. After centrifuging to throw down the larger particles, the supernatant is passed through a sterile Seitz filter (E.K.), giving a pale yellow filtrate which is stored in closed sterile flasks under refrigeration until used. In our experience the mixture, even when sterile, becomes opalescent and unsuited for use after about two weeks.

II. Test for alpha toxigenicity of strains of Cl. welchii. An 18-hour meat-tube culture of the strain to be tested is filtered through an L3 Pasteur-Chamberland filter candle. Using sterile technique, 0·5 c.c. of the filtrate is mixed with 0·5 c.c. of the lecitho-vitellin mixture in a sterile Wassermann tube. Two controls are set up : one of 0·5 c.c. of the filtrate mixed with 0·5 c.c. of physiological saline, the second of 0·5 c.c. of sterile meat-tube broth with 0·5 c.c. of lecitho-vitellin mixture. Test and controls are then incubated at 37° C. and hourly readings of alpha toxin levels are made until maximum reaction is reached, as follows :

Reaction	Alpha toxin level	Alpha toxin titre
No difference from controls	None	—
Opalescence	Minimal	+
Opalescence and turbidity	Low	+ +
Heavy turbidity	Moderate	+ + +
Separation : scum over clear fluid	High	+ + + +

III Test for inactivation of Cl welchii alpha toxin by antiserum (estimation of levels of alpha antibodies in unknown sera) 1 0 5 c c of filtered 18 hour meat tube culture of a known virulent strain of *Cl welchii* is mixed in a sterile Wassermann tube with 0 5 c c of the serum to be tested   One control of 0 5 c c of the culture filtrate with 0 5 c c of sterile broth and a second control of 0 5 c c of the culture filtrate with 0 5 c c of univalent *Cl welchii* antitoxin are also set up   The test and two controls are incubated overnight at 37° C

2 0 1 c c of the above test solution is added to 0 9 c c of lecitho vitellin mixture for the test   The same is done with each of the controls and they are all further incubated for 18 hours at 37° C   Readings are then made as follows

Unknown serum	Broth control	Antitoxin control	Alpha antibody level	Alpha antibody titre
—	+ + + +	—	= high	+ + + +
+	+ + + +	—	= moderate	+ + +
+ +	+ + + +	—	= low	+ +
+ + +	+ + + +	—	= very low	—
+ + + +	+ + + +	—	= none	—

— equals no opalescence and + to + + + + are grades of opalescence through turbidity and separation as in the alpha toxin end point readings

Thus controlled any alteration from the controls can certainly be attributed to alpha toxin lecithinase activity

Results

Using the above techniques the alpha toxigenicity of 28 strains of *Cl welchii* and 37 strains of other clostridia was tested   Of these strains 25 (2 *Cl welchii* and 23 other clostridia) were obtained from the laboratory of Dr Ivan C Hall, Presbyterian Hospital, New York City   The remaining strains (26 *Cl welchii* and 14 other clostridia) were isolated and identified in this laboratory from patients in this hospital   The results, which represent many repeated independent tests, are shown in table I

In the early part of the experiments, when the tests were set up without aseptic technique, a number of quasi positive tests (mild opalescence) were encountered   The opalescence was of a degree that might be expected merely from bacterial growth in the test solutions and was completely eliminated by the use of aseptic techniques   A second difficulty was encountered when one of the strains of *Cl tetani* persisted in giving a strongly positive reaction   This could be prevented by the addition of specific *Cl welchii* antitoxin but not *Cl tetani* antitoxin and was found to be due to contamination with *Cl welchii* When the culture was purified no positive reaction could be obtained

Of the 28 strains of *Cl welchii*, 25 gave readings indicating high levels of alpha toxin   Some of these reactions were delayed and all could be completely inhibited by specific *Cl welchii* antitoxin but were uninfluenced by specific *Cl tetani* antitoxin   With the exception of one strain each of *Cl botulinum* A and B, all other clostridia tested

gave negative reactions. These included 9 strains of the *sporogenes-bifermentans-sordelli* group. The strongly positive reactions of the

TABLE I

Alpha toxigenicity of clostridia

Organism	No. of strains tested	Alpha toxin level	Time to maximum reaction (hours)
Cl. welchii	15	+ + + +	3
,, ,,	5	+ + + +	4
,, ,,	1	+ + + +	5
,, ,,	1	+ + + +	6
,, ,,	3	+ + + +	18
,, ,,	1	+ +	18
,, ,,	2	—	18
Cl. botulinum A	1	+ + + +	4
,, ,, B	1	+ + + +	3
Cl. tertium	5	—	18
Cl. bifermentans	4	—	18
Cl. sordelli	3	—	18
Cl. septicum	2	—	18
Cl. sporogenes	2	—	18
Cl. histolyticum	2	—	18
Cl. putrificum	2	—	18
Cl. tetani	2	—	18
Cl. multifermentans	2	—	18
Cl. filamentosum	1	—	18
Cl. tetanomorphum	1	—	18
Cl. novyi	1	—	18
Cl. sphenoides	1	—	18
Cl. fallax	1	—	18
Cl. unidentified	6	—	18

2 strains of *Cl. botulinum* could not be inhibited by either specific *Cl. welchii* or *Cl. tetani* antisera. No specific *botulinum* antisera were available.

Table II shows the correlation of levels of alpha toxin as determined by the lecitho-vitellin technique and mouse toxicity. The mice were

TABLE II

Correlation of alpha toxin level and mouse toxicity in strains of Cl. welchii

No. of strains	Alpha toxin level	Time to maximum reaction (hours)	Mouse toxicity *
13	+ + + +	3-6	+
2	+ + + +	3-6	—
1	+ + + +	18	+
1	+ + + +	18	—
1	+ +	18	—
2	—	18	—

* + = mouse died in 18-36 hours. — = mouse survived 48 hours or longer.

injected intramuscularly with 0·2 c.c. of the culture, both with and without 0·1 c.c. of 2½ per cent. CaCl₂. Correlation is not absolute ; not all strains with an indicated high level of alpha toxin are uniformly

toxic for the mouse. However, there is confirmation of Hayward's observation that no strain with a negative reaction is toxic for the mouse. Further, the large majority of strains giving a strongly positive reaction early are toxic. Thus, like the Nagler reaction, this test gives false positive lethal alpha toxigenicity readings but never false negative readings. Actually, because it is more sensitive than the Nagler reaction, its proportion of false positives should be higher. In clinical practice, therefore, if a strain gives a negative reaction one may be justified in dismissing it as non-toxigenic so far as alpha toxin is concerned.

Sera from 36 dogs and cats were tested for alpha antibody levels using the described technique and the results are listed in table III.

TABLE III

Alpha antibody titres in unknown sera

Serum	Test serum reading	Broth control reading	Specific antitoxin control reading	Alpha antibody level
Dog				
1	+	+ + + +	—	+ + +
2	+	+ + + +	—	+ + +
3	+	+ + + +	—	+ + +
4	+	+ + + +	—	+ + +
5	—	+ + + +	—	+ + + +
6	—	+ + + +	—	+ + + +
7	+ + +	+ + + +	—	+
8	+	+ + + +	—	+ + +
9	+ +	+ + + +	—	+ +
10	+ +	+ + + +	—	+ +
11	+ + +	+ + + +	—	+
12	+ + +	+ + + +	—	+
13	+ +	+ + + +	—	+ +
14	+ +	+ + + +	—	+ +
15	+ +	+ + + +	—	+ +
16	+ + +	+ + + +	—	+
17	+ + + +	+ + + +	—	—
18	+ +	+ + + +	—	+ +
19	+ + + +	+ + + +	—	—
20	+	+ + + +	—	+ + +
21	+ + + +	+ + + +	—	—
22	+	+ + + +	—	+ + +
23	—	+ + + +	—	+ + + +
24	—	+ + + +	—	+ + + +
25	—	+ + + +	—	+ + + +
26	—	+ + + +	—	+ + + +
27	—	+ + + +	—	+ + + +
28	—	+ + + +	—	+ + + +
29	—	+ + + +	—	+ + + +
30	—	+ + + +	—	+ + + +
31	+ + +	+ + + +	—	+
32	—	+ + + +	—	+ + + +
33	+	+ + + +	—	+ + +
34	+ +	+ + + +	—	+ +
Cat				
1	—	+ + + +	—	+ + + +
2	—	+ + + +	—	+ + +

It had been our experience that these animals had rather high natural alpha antibody titres and they were considered ideal test animals.

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injected intramuscularly with 0·2 c.c. of the culture, both with and without 0·1 c.c. of 2½ per cent. CaCl₂. Correlation is not absolute; not all strains with an indicated high level of alpha toxin are uniformly

ADRENAL TUMOURS AND PSEUDO-HERMAPHRODITISM: A HORMONE STUDY OF CASES *

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(PLATES XXX AND XXXI)

IN recent years the adrenal cortex has tended to usurp the position formerly held by the anterior pituitary as the secreter of an ever-increasing list of substances Among these, attention has been directed to the sex hormones, which have been not only isolated from the gland itself but also recovered from the urine of persons suffering from abnormal function of the adrenal cortex The two chief groups of substances referred to are progesterone (excreted as pregnanediol) and androgens (excreted as 17-ketosteroids) , the cortex is also a source of œstrogen Disturbances in cortical function, whether due to hyperplasia or neoplasia, are accompanied by increased secretion of cortical hormones, particularly androgens These give rise to that inhibition of feminine characters and functions which constitutes the clinical picture of virilism—hypertrichosis of male type, menstrual dysfunction, and changes in bodily contour, external genitalia, larynx and psychological outlook The development of some or all of these symptoms after maturity is of not infrequent occurrence, and the category of those exhibiting manifestations of that indefinite entity called " the adreno genital syndrome ", in which the androgen output is only moderately raised, is a large one A report of such cases will be made at a later date The present study is confined to cases of adrenal cortical tumour and to patients with disordered cortical function occurring not at or after maturity but ante natally, so as to affect sex development at the outset This group comprises male and female pseudo hermaphrodites, the former with the gonads of the male, the latter with those of the female

If the clinical picture of virilism were in direct relation to the extent or type of the cortical lesion the clinician's task would be easy , as it is, it is frequently difficult for him to assess at their proper value the symptoms of virilism in women as presented to him An adrenal tumour may exist in the absence of marked or even of any

hirsutism, whereas severe hypertrichosis may be associated with apparently normal adrenal secretion. The existence of morphological abnormalities of the sexual organs, sometimes without other evidence of virilism, calls for differentiation between an error in diagnosis of sex (*e.g.* a male pseudo-hermaphrodite) and the presence of either adrenal hyperplasia or an adrenal tumour. In all such cases a knowledge of the excretion of the 17-ketosteroids (*i.e.* the urinary androgens) can, as a rule, be of great help to the clinician. It is not to be inferred that hypertrichosis is solely an accompaniment of adrenal anomaly, as it has been found in a variety of unrelated conditions. It has also been reported in patients with Cushing's syndrome whose adrenals have been shown to be normal both by a normal to low excretion of 17-ketosteroids and by histological examination after autopsy. As a measure of the adrenal involvement in this condition, the 17-ketosteroids output provides information essential to diagnosis.

Since the earlier report of a case of adrenal cortical carcinoma (Anderson *et al.*, 1943) the hormonal investigation of patients with symptoms of virilism has been productive of 6 other cases in which adrenal cortical tumours were verified at operation, two of them males. In addition eight patients are described whose symptoms suggested either tumour or marked hyperplasia of the adrenal cortex. Two of these were sisters and in two others one or more members of the family were similarly affected. In three instances an error was made in diagnosis of sex and in only one of these has cortical hypersecretion persisted. In all cases the excretion of the androgens, of which the 17-ketosteroids are the degradation products, was estimated; where possible the output of pregnanediol (the excreted form of progesterone) and of gonadotropin was also ascertained, and, in a few cases, of œstrogen.

There has been a clamant need for rapid and inexpensive methods of estimating the chief hormones excreted in the urine; in the absence of a chemical test directly applicable either to the patient or to the untreated urine, the methods of extraction and assay to be described are in both respects a great advance on those hitherto in use. By Patterson's method it is possible to estimate the 17-ketosteroids output within 24-30 hours of receiving a small sample of urine; by using kaolin instead of alcohol to precipitate the gonadotropin in urine a great saving of expense is effected; unfortunately the recovery of pregnanediol is still somewhat lengthy and the assay of œstrogens remains long and arduous; only for estimation of the 17-ketosteroids are large amounts of urine not required.

METHODS

17-Ketosteroids. The method of hormone extraction prior to colorimetric assay was that used by Patterson *et al.* (1942), who generously provided me with the details, previously unpublished. Determinations were made on a 24-hour basis and not per litre of urine, only 20 c.c. being required for analysis.

This was taken from the bulk of a complete 24-hour specimen preserved with toluene, a note of the total volume being sent with the small sample. The 20 c.c. was hydrolysed by boiling under a reflux condenser for 30 minutes with 0·6 c.c. concentrated sulphuric acid and 2·5 c.c. pure toluene. The cooled urine was extracted twice with 25 c.c. lots of peroxide-free A.R. ether. The ethereal extracts were purified by being washed twice with 3·0 c.c. of a 10 per cent. solution of sodium hydroxide and twice with the same amount of a freshly prepared reducing agent consisting of 2 g. of sodium hydrosulphite ($Na_2S_2O_4$) in 20 c.c. of 0·1 N sodium hydroxide. Each washing consisted of vigorous shaking with the ethereal extract for three minutes before allowing the chemical separation. The ether was washed free of alkali and reducing agent with three lots of 3 c.c. distilled water, after which it was transferred to a boiling-tube containing a knife-point of Norit charcoal and evaporated slowly at a temperature not exceeding 42° C. When reduced to 7 or 8 c.c. the ether was filtered into an evaporating flask through a filter made from a drawn-out test-tube into which a plug of cotton wool was fitted: 5 or 6 c.c. of fresh ether were boiled up with the Norit charcoal in the boiling-tube and used as wash liquid. The ether and toluene were carefully evaporated as before, the latter with the aid of a current of air from a bulb-type bellows fitted with a pipette containing a plug of cotton wool to exclude dust. The dry residue was transferred to a 15 c.c. test-tube with two or three washings of ether, the ether slowly evaporated off and the residue dissolved in 0·5 c.c. pure absolute alcohol.

This alcoholic fraction was used for the colorimetric assay of the 17-ketosteroids; these were determined quantitatively by the use of *m*-dinitrobenzene, which gives a red colour in the presence of alkali with compounds containing an active methylene group. The colour produced in the reaction has a characteristic absorption band in the green and the absorption light in this region of the spectrum, measured on a Hilger model Spekker photo-electric absorptiometer with a green filter, was taken as a measure of the 17-ketosteroids present in the urine. The *m*-dinitrobenzene used was B.D.H.'s " specially purified ", M.P. 91·92°, which requires to be further purified by the method described by Callow *et al.* (1938).

For colour development 0·2 c.c. of the alcoholic solution was transferred to a 15 c.c. test-tube, 0·2 c.c. of 2 per cent. *m*-dinitrobenzene in absolute alcohol was added, followed by 0·1 c.c. of a freshly prepared solution of 2·5 N potassium hydroxide in absolute alcohol. Dr Patterson used an aqueous solution of 4 N KOH instead of the alcoholic solution of 2·5 N. Whichever method is adopted for the calibration curve must be adhered to for the unknowns. The test-tube was quickly and tightly stoppered and, after being shaken to mix the reagents, was placed in a water-bath maintained at 25° C. for one hour. A blank was run at the same time with 0·2 c.c. absolute alcohol and the same quantities of reagent as for the unknowns. From the time of adding the reagents to the tubes these were shielded from all but dull diffused light. Immediately before reading in the Spekker absorptiometer, 10 c.c. of absolute alcohol were added to each tube and the contents mixed. Each reading should be completed within 5 minutes of diluting with alcohol. The ketosteroids value was calculated from a calibration curve constructed from standard solutions of androsterone covering the range 25-200 gamma per 0·2 c.c. Dilutions of the alcoholic extract were made when it was necessary to bring the colour intensity within the range of the calibration curve.

At the conclusion of every estimation test-tubes used for colorimetry must be cleaned with nitric and chromic acid mixture, carefully rinsed and dried in a steriliser.

Pregnanediol. The gravimetric method used was that of Venning (1938).

Gonadotropin. Instead of expensive precipitation with alcohol, a modification of the kaolin method used by Scott (1940) for the concentration and

hirsutism, whereas severe hypertrichosis may be associated with apparently normal adrenal secretion. The existence of morphological abnormalities of the sexual organs, sometimes without other evidence of virilism, calls for differentiation between an error in diagnosis of sex (*e.g.* a male pseudo-hermaphrodite) and the presence of either adrenal hyperplasia or an adrenal tumour. In all such cases a knowledge of the excretion of the 17-ketosteroids (*i.e.* the urinary androgens) can, as a rule, be of great help to the clinician. It is not to be inferred that hypertrichosis is solely an accompaniment of adrenal anomaly, as it has been found in a variety of unrelated conditions. It has also been reported in patients with Cushing's syndrome whose adrenals have been shown to be normal both by a normal to low excretion of 17-ketosteroids and by histological examination after autopsy. As a measure of the adrenal involvement in this condition, the 17-ketosteroids output provides information essential to diagnosis.

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Hormone findings. 17-Ketosteroids were 44-45 mg./24 hours (80-100 mg./litre) during the four months before the patient died. The average daily output of a normal adult women is 7·4 mg. and in cases of adrenal tumour generally over 70 mg.; Callow's lowest figure was 40 mg. in a man with an adrenal tumour. The small urinary output in this case, due to renal damage, was undoubtedly responsible for the relatively low androgen excretion. This fell to 20 mg. on the day before death, when the patient was passing only a few ounces of urine.

Pregnanediol was continuously excreted (25 mg./24 hrs.) in spite of the fact that the patient menstruated twice during this time. Actually while menstruating, four months before she died, she excreted 15 mg. of pregnanediol per litre. On the day before her death, owing perhaps to faulty formation of the glucuronide or to inability of the adrenal cortex to metabolise progesterone, large amounts of a pregnane derivative were recovered instead of sodium pregnanediol glucuronidate; in the acetone fractions in Venning's method many colloidal masses formed instead of the usual white powder.

Œstrogen and Aschheim-Zondek test. Both tests were negative when 3 c.c. of untreated urine were used, indicating in the case of the former that adrenal carcinoma is not always associated with the high œstrogen output which Frank (1933-34, 1937), Graef *et al.* (1936), Lukens and Palmer (1940), Gross (1940), McGavack (1940) and others have encountered. A negative finding for œstrogen was obtained also in the case of Anderson *et al.* (1943) and in two others reported here.

Post mortem, a tumour in the region of the left adrenal measuring $10\frac{1}{2} \times 7\frac{1}{2} \times 5\frac{1}{2}$ in. and weighing 4270 g. (approx. $9\frac{1}{4}$ lbs.) was found. There were extensive secondaries in the lungs, liver and left kidney; the left lung weighed 700 g., of which half was tumour tissue. The left ovary was also involved; unfortunately the uterus was not examined.

Histology (figs. 1 and 2). The tumour was examined by Professor Blacklock, Dr Helen Russell and Col. Harvey. It was highly cellular, but with very indefinite characters. There was great cellular polymorphism, and monster cells, both mono- and multinucleated, and monster hyperchromatic nuclei were striking features. In places the tumour was imperfectly adenomatous in character, but spheroidal cells predominated and there were numerous mitoses. Dr Russell expressed the opinion that it was a malignant tumour of primitive gonadal type arising from some part of the genital ridge. She found a somewhat similar structure in the left ovary, which had appeared normal macroscopically. It contained in addition many immature follicles and a few of larger size which showed a hyperplastic granulosa-cell layer. There appeared to be a mixture of both male and female elements and some resemblance to arrhenoblastoma. Col. Harvey suggested that the growth was an adrenal carcinoma. Professor Blacklock noted a marked increase in the eosinophil cells of the pituitary.

Commentary. The results of hormone analysis were such as to place it beyond doubt that this patient's tumour was an adrenal cortical carcinoma or virilising gonadal tumour, and it would seem that histologically it might be regarded as an adrenal tumour of undifferentiated cortical tissue such as is found in malignant cortical tumours associated with the adreno-genital syndrome. The following paragraph by Geschickter (1935, p. 112) is relevant to the condition found in both the adrenal and the ovary in this case. " The origin of the suprarenal cortex from the genital ridge relates these un-differentiated cells in the embryo to the gonocytes. If the explanation of Krabbe is accepted, that the testis takes its origin directly from such undifferentiated tissue, while the ovary represents a more highly differentiated structure, then the adrenal cortical tissue in its more primitive state is identical with testicular tissue ". Indeed, Krabbe (1921) thinks that cortical tumours develop from male sex gland cells which, early in embryonic life, become involved in the adrenal cortex and produce the adreno-genital syndrome.

In this connection the observations of Fekete and Little (1945) are significant. Carcinoma of the adrenal cortex occurred in a high percentage of mice of the " ce " strain after gonadectomy; there were no tumours in the intact mice of either sex. In two mice in which ovarian regeneration occurred, the nodule was composed mainly of atypical cells forming an ovarian carcinoma which bore a striking resemblance to the adrenal carcinoma structurally. The action of adrenal carcinoma in mice is chiefly feminising, as it causes œstrus and mammary gland development equal to that in an intact mouse of the same age (Woolley and Little, 1945).

Certain features of the present case deserve comment.

1. Menstruation occurred four times during the last eight months of the patient's life, the last occasion being only a month before the patient died. That menstruation continued in spite of the existence of an adrenal tumour and of the excretion of large amounts of ketosteroids and pregnanediol, each of which would have been thought sufficient to arrest menstruation, is remarkable. There is only one other case known to the author of menstruation in an adult with an adrenal tumour, namely that of Kepler *et al.* (1934), a woman aged 25 who menstruated regularly until two months before removal of a cortical tumour measuring 12 × 10 cm. The occurrence of vaginal bleeding in children with adrenal tumours is not infrequent, five instances having been recorded up to 1938 (Bulloch and Sequeira, 1905; Cecil, 1933; Kepler *et al.*, 1934 and 1938; Walters and Kepler, 1938). Numerous instances are on record of its persistence (though irregular) in very hirsute women in whom marked adrenal hyperplasia was present. Shepardson and Shapiro (1939) give a striking example.

The possibility that the continuance of menstruation in the present case was attributable to the tumour being primarily one of the lung

is regarded as unlikely in view of the size of the adrenal growth and the hormone output. That the abnormal ovarian condition may have been partly responsible is suggested by a case described by Mechler and Black (1943). This patient, aged 24, complained of virilism of five years' duration ; she had a deep voice, beard, acne and enlarged clitoris. Her periods, regular since the age of 14, had occurred every two weeks for 5-7 days during the past four months and vaginal bleeding had lasted ten days prior to the removal of an ovarian tumour, 11 cm. in diameter, which contained elements of both a granulosa-cell tumour and an arrhenoblastoma. That menstruation occurred simultaneously with the excretion of large amounts of pregnanediol demonstrates that the menstrual rhythm is not due to cessation of progesterone secretion. It might be contended that the progesterone secreted by an adrenal tumour is perhaps of different chemical structure from that secreted by the ovary ; this is unlikely, as Dr Patterson recovered the expected amount of pure pregnanediol from samples of the sodium pregnanediol glucuronide from this patient.

Various authors (Herlant, 1939 ; Burrows, 1939 ; Biddulph *et al.*, 1940) have demonstrated that progesterone curtails the supply of gonadotropin from the pituitary of rats, yet it is clear that, in our case, progesterone was unable to inhibit the mechanism controlling the menstrual rhythm, whether such mechanism resides in the pituitary or the ovary. Since menstruation is a pseudo-parturition it is pertinent to point out that parturition occurs in spite of the secretion of large quantities of progesterone, as evidenced by the recovery of 30-50 mg. of pregnanediol during labour (Hain, 1942-44). So also menstruation in the normal non-pregnant woman can take place in spite of the excretion of amounts of pregnanediol commonly associated with the " secretory phase " in the endometrium (Hain and Robertson, 1939). Furthermore, cases are reported of persistence of this type of endometrium after menstruation, from which Wilson and Kurzrok (1940) formed the opinion that the pituitary is not an immediate factor in the production of menstrual bleeding.

Another possible explanation is that menstruation occurred as the result of periodic drops in the level of œstrogen secreted. The excretion of œstrogen in a combined form was not ascertained, but injection of the untreated urine into mice was without effect and showed that the amount secreted by the tumour was not excessive. Case 13 in this series shows that a high œstrogen secretion combined with a high progesterone secretion is not necessarily accompanied by the establishment of the menstrual rhythm or the development of other secondary sex characters.

2. There was excretion of a pregnane derivative other than pregnanediol. That this occurred only when the patient was moribund and after normal pregnanediol had been recovered over a considerable period suggests faulty function either of the adrenals or of the kidneys. The same substance has been recovered by the author in a

variety of conditions besides virilism—in two women with mastitis, in two men with gynecomastia, and in a pregnant woman with virilism in whom the abnormal substance and the normal glucuronide were precipitated in the same acetone fraction. In spite of very inadequate recovery from the acetone, sufficient material was collected to warrant further investigation, which Professor Marrian kindly undertook, but wartime duties prevented its completion. The excretion in cases of virilism of compounds related to the steroids of the adrenal cortex has been reported by Butler and Marrian (1938), Wolfe et al. (1941), Strickler et al. (1943), Dorfman et al. (1945) and Mason and Kepler (1945).

3. Ovarian involvement in a case of adrenal tumour is not unique. Similar cases have been reported by Tuffier (1914), Jeanneney and le Calvez (1938) and Norris (1938), while a case of testicular involvement is recorded by Wilkins et al. (1940). An interesting case of an adrenal cortical rest in the pancreas is cited by McLetchie and Scott (1942-44, p. 351), who think it likely that the associated adrenal cortical hyperplasia was produced by the same stimulus as induced the malignant change in the adrenal rest. Such an explanation might apply to our case 1.

4. Hypertension in patients with adrenal cortical tumours or hyperplasia is the rule rather than the exception. Nuzum and Dalton (1938) collected records of 29 cases of virilising tumours showing this association and the number has doubled since that date, no fewer than seven being in children. The hypertension of virilism is not solely or directly of adrenal cortical origin ; it occurs also in cases of virilising tumours of the ovary (Kepler et al. (1944) cite 4 cases in addition to their own), in testicular tumours (one such case is soon to be published), and in granulosa-cell tumours of the ovary (Gross ; a girl aged 13 months). In all these, the cells involved are derived from the same anlage as the adrenal cortex and their secretions have similar effects on processes ultimately controlling vascular tension.

5. In Professor Blacklock's opinion the most interesting feature of the case was the marked increase in pituitary eosinophil cells, not of basophils as might be expected from the association of basophil adenomas or hyalinisation (Crooke's cells) with adrenal cortical tumours. This excess of eosinophils has been observed on several occasions in association with adrenal cortical hyperfunction (Mathias 1922 ; Oppenheimer and Fishberg, 1924 ; Long and Gray, 1924; Josephson, 1936 ; Gross, 1940, case 4). Cushing and Davidoff (1927) reported that in each of four cases of acromegaly associated with acidophilic pituitary adenomas the adrenals were hypertrophied, the largest being four times the normal weight. In addition Driggs and Spatze (1939, quoted by Weinberger and Grant, 1941) have described the case of a boy, aged $3\frac{1}{2}$, in whom sexual precocity was due to a tumour suspended from the third ventricle and in whose pituitary a predominance of eosinophil cells was found. Evidence that both

ADRENAL TUMOURS AND PSEUDO-HERMAPHRODITISM

FIG. 1.—Case 1. Section of primary adrenal carcinoma in a girl aged 21. ×400.
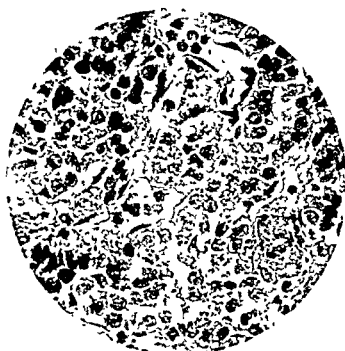
FIG. 2.—Case 1. Section of metastatic deposit in lung, showing adrenal cortical structure, with some cells of giant size. ×400

FIG. 3.—Case 2. Patient aged 3½ years with hirsutism, adiposity and premature skeletal development due to an adrenal tumour.

types of cell—basophil and eosinophil—take part in the hypophyseal control of gonadal activity is provided by the fact that castration, thyroidectomy and the administration of hormones have their effect on both. It is possible, therefore, that adrenal hyperfunction can cause an excess of either type of cell in the pituitary.

6. The weight of the primary tumour (9¼ lb.) is exceeded by one weighing about 15 lb. reported by Baldwin (1914) in a boy aged 6, and by one of some 20 lb. in a woman of 36 recorded by Glynn (1911-12).

Case 2. Miss Herzfeld's case (P 323), a girl aged 3½ years (fig. 3). Long downy hair over her whole body was noticed at the age of 12 months and became thicker, especially over the pubes, thighs and back of the shoulders; the clitoris was large, there was acne and a deep voice; the features were heavy and she was full-coloured. There was no real obesity but she was 8 lb. above the average weight for her age. The mammæ were not overdeveloped, there were no striæ or osteoporosis. The pituitary fossa was normal. Ossification was that of a child of 8-9 years. There was polycythæmia (R.B.C. 7·8 million) and hypertension (B.P. 128/88) and her Hb. was 107 per cent.

Hormone findings. 17-Ketosteroids 5·9 mg./24 hours (analysis by Dr Patterson); pregnanediol 9, 12·5 and 7·5 mg./24 hours, Aschheim-Zondek and œstrogen tests negative with 3·0 c.c. of urine. The ketosteroids were not abnormally high as compared with the daily output of a normal child of the same age, namely 1·3 mg. (Talbot *et al.*) or 1·4 and 2·5 mg. in our own controls; they were exceedingly low as compared with those in case 3. The high pregnanediol output, however, pointed definitely to marked hyperactivity of the adrenal cortex and a diagnosis of adrenal anomaly was made on this ground alone, as the only other occasions on which pregnanediol is recoverable are in pregnancy and in the luteal phase of the menstrual cycle. Its association with cortical hyperplasia and tumour has been demonstrated by Venning *et al.* (1939), Salmon *et al.* (1941) and Anderson *et al.* (1943).

At laparotomy the right adrenal was found to be almost completely replaced by a spherical tumour about 2⅓ inches in diameter. It was well encapsulated, reddish on section, but neither hæmorrhagic nor necrotic. The uterus and ovaries were larger than normal for this age. It was decided to operate on the adrenal tumour at a later date and only the appendix was removed. The patient died of streptococcal peritonitis a few days later. At autopsy, secondary deposits were found in the lungs. Histologically the adrenal tumour consisted of closely packed solid columns of cells with a delicate supporting stroma carrying the blood vessels. The cells, which were rounded or polygonal, resembled the cells and had the general arrangement of suprarenal cortical tissue. The structure of the pulmonary secondaries was similar, and the case was regarded as one of adrenal cortical carcinoma with metastasis to the lungs.

Case 3. Dr McCall's case (K 30), a girl aged four years. Pubic hair, noticed for 10 months, had become abundant. The patient was $6\frac{1}{2}$ in. above the average height and $10\frac{1}{2}$ lb. above the average weight for her age; ossification was that of a child of 12 years; Hb. 81 per cent., R.B.C. 4·2 million; glucose tolerance normal. The clitoris was considerably enlarged; she looked older than her age and more intelligent, but was tired and listless and complained of vague abdominal pain.

Hormone findings. 17-Ketosteroids, estimated over 24 hours on two occasions, were 320 mg. (375 mg./litre) and 166 mg. (322 mg./litre). No pregnanediol was recovered on either occasion, an interesting finding in view of the enormous amount of 17-ketosteroids excreted and in direct contrast with the hormone output of case 2. Œstrogen and gonadotropin were less than 5 mouse units. Almost three weeks after removal of the tumour the 17-ketosteroids output had fallen to 3·7 mg./litre.

It was thought that a cytological difference might be discernible between this tumour and that of case 1, which was secreting considerable amounts of progesterone, but no difference was detected histologically. Goormaghtigh (1940) claims a cytological basis for the difference between feminising and virilising tumours of the adrenal cortex.

The tumour, which was on the left side and encapsulated, weighed 38·6 g. and measured $5·7 \times 3·9 \times 2·9$ cm. It showed distinct lobulation and was soft and cystic, with cavity formation at one end. Histologically it was pronounced to be a benign cortical adenoma; mitoses were rare but large hyperchromatic nuclei were present and a positive fuchsin-ponceau reaction was obtained.

Case 4. Mr Broster's case (K 125), a boy aged 17 years, submitted as a case of Cushing's syndrome for information as to adrenal involvement. The urine was four days' old when received but in spite of this a ketosteroids output of 49·1 mg./24 hours was obtained. An adenoma " the size of an acorn " was found in the substance of the right adrenal.

Case 5. Dr R. B. Magill's case (K 148), a boy aged 10 years and 10 months, but with the muscular development and physique of a man of 18 (fig. 4). His beard hairs were $\frac{3}{4}$ in. long, pubic hair was very profuse, and his arms, legs and axillæ were as hairy as those of a fully-grown man. His testicles were normal for his age but his penis was very large. Radiologically his hands were those of a boy of 12, his intelligence that of a boy of 11; height was 61 in., weight $124\frac{1}{2}$ lb. His pituitary fossa was reported as small.

Hormone findings. 17-Ketosteroids, 84 mg./24 hrs.; pregnanediol none.

When the patient was aged $5\frac{1}{2}$ years a laparotomy revealed a tumour over the left kidney and about $\frac{1}{2}$-$\frac{1}{3}$ its size. No attempt was made to remove it. The sister of this patient, aged $3\frac{1}{2}$ years, exhibits

the usual symptoms of prepubertal virilism and undoubtedly has either an adrenal cortical tumour or hyperplasia. Her case is described in the next section (case 8), as laparotomy has not yet been performed.

The occurrence of adrenal tumours or hyperplasia in more than one member of a family is not rare; 10 cases additional to his own were collected from the literature by Wilkins *et al.* To these may be added one family each reported by Broster (1934) and Rhodes (1943) (in both of which twins were affected), Guthrie and Emery (1907) and Miller and Kenny (1939-40), as well as the three families here described and the case reported by Kepler *et al.* (1944) in which a virilising ovarian tumour was removed from a girl whose sister presented the same picture. If this is included, there are approximately 20 instances of virilising growths occurring in more than one member of a family. This takes no account of a wider relationship.

Instances of adrenal cortical tumours in boys are rare and as only 23 other cases have been found in the literature they are listed in table II. In one case (Holl, 1930) the tumour had a feminising effect. Adrenal cortical hyperplasia in boys is even rarer and only two cases are on record (Hutchison, 1904; Geschickter, 1935) with a possible third in the case of Jacobziner and Gorfinkel (1936).

TABLE II

Adrenal cortical tumours in boys

Year	Author	Age	
1903	Linser (quoted by Gordon and Browder, 1927)	5 yr.	7 mth.
1905	Adams	14 yr.	9 mth.
1907	Guthrie and Emery	4 yr.	4 mth.
1914	Baldwin	5 yr.	10 mth.
1919	Tschernobrow (quoted by Gordon and Browder)	11 yr.	
1927	Gordon and Browder	3 yr.	
1928-29	Fordyce and Evans	2 yr.	3 mth.
1929	Macera	3½ mth	
1930	Holl	15 yr.	
1931-32	Harwood	7 yr.	
1932	Lightwood	18 wk.	
1933	Rowntree and Ball	30 mth.	
1933	Player and Lisser	4 yr.	11 mth.
1934	Nobécourt	8 yr.	
1935-36	Mainzer	8½ yr.	
1936	Jacobziner and Gorfinkel	4 yr.	9 mth.
1936	Josephson	17 yr.	
1937-38	von Kup	3½ yr.	
1939-40	Fraser	1 yr.	
1940	Gross	3 yr.	
1940	Wilkins *et al.*	3 yr.	7 mth.
1942	Patterson *et al.*	5 yr.	
1945	Broster (personal communication)	11½ yr.	

Case 6. Dr Steven Faulds's case (P 265), a woman aged 60. The tumour, first thought to be an arrhenoblastoma of the ovary, was, in the opinion of Professor M. J. Stewart, an ectopic adrenal cortical tumour. The patient had a markedly enlarged clitoris, a very heavy

growth of hair on the face, shoulders and chest, and a deep voice. Unfortunately urine was not submitted for analysis before· removal of the growth ; a post-operative specimen contained the equivalent of 7·7 mg. 17-ketosteroids/24 hours. The exudate from the tumour was reported by Dr Patterson to contain only 15 gamma free keto-steroids and 50 gamma bound ketosteroids in 19 c.c. of fluid, indicating that very little of the androgen is stored in the tumour, which is in agreement with the general finding.

, In two other cases of adrenal neoplasm, each of which proved to be a neuroblastoma, a knowledge of the excretion of 17-ketosteroids proved of value in excluding a cortical tumour ; in both, the excretion was normal in amount in spite of the presence of a tumour of considerable size depressing the kidney. Metastases to the bones had occurred. The patients were a girl aged 13, excreting 8·8 mg. 17-ketosteroids/24 hours, and a boy aged 2½, whose output was 1·4 mg./24 hours or 4 mg./litre. Aschheim-Zondek tests were negative.

GROUP II. PSEUDO-HERMAPHRODITES AND DOUBTFUL CASES OF ADRENAL TUMOUR

The second group of cases consists of patients regarding whose sex there was doubt at birth owing to the presence of morphological characters of both sexes, and in whom symptoms of virilism or absence of feminism developed. It has been found desirable to adopt this narrow interpretation for the term pseudo-hermaphroditism and to group under " adreno-genital syndrome " the numerous cases in which symptoms of virilism first manifested themselves at a later date. In some of the cases which follow, the existence of an adrenal cortical tumour seems likely, partly on account of the amount of hormone excreted, but also because a tumour occurred in another member of the family. They are placed in this group because a laparotomy has not been performed or did not reveal a tumour.

Case 7. Miss Herzfeld's case (K 98), a girl aged 2½ years and weighing 3 st., had an enlarged clitoris. Two sisters aged 17 and 19 had the same abnormality and from one of these Miss Herzfeld removed a cortical tumour. There were two brothers, both normal. 17-Ketosteroids 21·8 mg./litre.

Case 8. Dr R. B. Magill's case (K 139), a girl aged 3½ years ; build equal to 6-7 years, with hands of a radiological age of 7-8 years. The clitoris was as large as the penis of a boy of 4 and there was some pubic hair, first noted at 2½ years. Ovaries and uterus were found under anæsthesia. Her brother (case 5) has an adrenal cortical tumour. 17-Ketosteroids 12 mg./24 hours or 16·3 mg./litre : pregnane-diol none.

Case 9. Dr McCall's case (K 85), a girl aged 1 year 11 months, weight 30 lb., height 33 in., had a very large clitoris, pubic hair (noticed for six months) and a normal vagina. She was backward in making talking noises and had a deep voice. 17-Ketosteroids 23·5 mg./litre.

No pregnanediol. At laparotomy no tumour was found, but both adrenals were thought to be enlarged.

The next two cases provide an interesting contrast as, in both, an initial error was made as to the sex, one being brought up as a boy and found to be a girl, the other contrariwise. Both were 17 years old at the time of the hormone investigation.

Case 10. Miss Herzfeld's case (K 73) was brought up as a boy until she was 9 years old, when ovaries and uterus were found at laparotomy, and a small vagina ; the enlarged clitoris was removed and a portion of the right ovary in which there was excess of fibrous tissue ; no adrenal tumour was found. Pubic hair of masculine type was well developed, but, apart from absence of mammary development, the patient had a female type of figure. When $11\frac{1}{2}$ years old she complained of growth of hair on the lip ; there was still no mammary development. Œstrin injections were begun at $12\frac{1}{2}$ and after six weeks the breasts were larger and the same course was repeated after three months. Albuminuria was $+++$, B.P. 160/90. When she was $14\frac{1}{2}$ years old the œstrin was increased and a preparation of pregnant mare's serum was given ; severe albuminuria persisted. Between the ages of 15 and $16\frac{1}{2}$ (*i.e.* in 16 months) she menstruated three times while taking stilbœstrol tablets. Menstruation was accompanied by urticarial attacks. B.P. was now 122/82. Breast development was equal to twelve years ; pubic hair was profuse. Though without œstrin for a month, she menstruated (from a hypoplastic endometrium) on April 1945 and five days before this period her daily hormone output was : 17-ketosteroids 62 mg., gonadotropin 7 mouse units (M.U.), pregnanediol none. The absence of excretion of pregnanediol might have been due to an absence of progesterone secretion, but it might also have been due to the seriously impaired kidney function. The ketosteroids output denoted either marked adrenal cortical hyperplasia or an adrenal cortical tumour. An operation could not be performed owing to the renal condition.

Case 11. Dr T. N. Macgregor's case (K 77) was brought up as a girl until the age of 17, when it was found that, in addition to a penis and a vagina, " she " had a testicle in each inguinal canal. Unfortunately permission to operate was not granted.

17-Ketosteroids 15·6 mg./litre (24 hours' output not known). This is not an abnormal figure for a youth of 17 and does not denote any appreciable hypertrophy of the adrenal cortex. It is in marked contrast to the output in the preceding case and to all others in this group with the exception of case 12 immediately following, and permits of a differential diagnosis as between male and female pseudo-hermaphroditism. In the latter, hyperfunction of the adrenal cortex persists and is responsible for the condition, manifesting itself in the excretion of excessive amounts of ketosteroids and sometimes of pregnanediol. In male pseudo-hermaphroditism, on the other hand,

the ketosteroids output is either normal or only slightly raised, the condition having arisen and been completed antenatally, as will be discussed later.

Young (1937, p. 292 *et seq.*) describes a case of this kind. A male pseudo-hermaphrodite, aged 26, had been brought up as a female ; the features were a minute penis 2 cm. long, a bifid scrotum, undescended testes 3 cm. long, and a tiny vagina 1·5 cm. long. There was no breast development and there had been no evidence of menstruation. Hair distribution was feminine and there was no hair on the face. Shoulders, arms and chest appeared masculine, and the features as well as the hands and feet were large. Body and extremities were without hair. The impression, after examination, was that it was impossible to make out any female organs except the minute vagina. Bodies, presumed to be testes, were present in the groins. A laparotomy was undertaken to settle the sex of the patient ; no uterus, tubes or ovaries were found but a small testis lay in each inguinal canal. As the patient wished to retain her female character an operation was performed to reconstruct the vagina. The testes were not removed but were placed within the abdomen as being the probable source of the œstrogen responsible for the female character of the patient.

Novak (1935) records a similar case, and Broster and Vines (1933) two, in one of whom the adrenals were slightly enlarged. In each case the testes were removed as, psychologically, the patient was dominantly female.

It is probable that the case which follows is really a male pseudo-hermaphrodite ; an operation is not to be performed meantime.

Case 12. Mr Linton Snaith's case (K 122), a girl aged eight years, had a hypertrophied clitoris but no other sign of virilism. 17-Ketosteroids were less than 2 mg./24 hours and only a trace of pregnanediol was recovered. A similar case is recorded by Simpson *et al.* (1936), namely that of a pseudo-hermaphrodite aged four years, whose urine contained no excess of androgens, whereas in two others these were very high. Case 12 was found to be excreting almost 20 M.U. of gonadotropin per 24 hours, which is as much as an adult woman excretes.

Cases 13 and 14. Mr Gemmell's patients (P 499 and K 42), two sisters aged 21 and 16 respectively, with primary amenorrhœa, had no other symptoms of virilism than an enlarged phallus (fig. 6) and absence of breast development. Their voices were not low nor was there any hirsutism ; outlook and figure were feminine. Permission was obtained to examine only the elder sister (fig. 5) and Mr Gemmell was unable to detect any uterine body or gonads in the pelvis or inguinal canals. Her blood pressure was normal (135/70). A still older sister has no abnormality.

With the ready cooperation of both girls hormone analyses were carried out over a prolonged period, the results of which are given in table III.

Owing to the high excretion of 17-ketosteroids and pregnanediol, a provisional diagnosis of adrenal tumour or bilateral hyperplasia of the adrenal cortex was made, but the possibility of an arrhenoblastoma was considered.

ADRENAL TUMOURS AND PSEUDO HERMAPHRODITISM

FIG 4—Case 5 (on right) aged 10 years and 10 months with precocious sexual and skeletal development due to an adrenal tumour his normal brother aged 13 years on left

FIG 5—Case 13 Patient aged 21 with primary amenorrhœa showing absence of breast development Hair distribution and figure are feminine Adrenal cortical hyperplasia was present

FIG 6—Same patient external genitalia, showing enlarged phallus

FIG 7—Same patient tiny vagina exposed

At operation infantile and completely inactive female genitalia were found but no male organs; there was a narrow vagina separate from the urethra (fig. 7). Both adrenals were considerably enlarged and the left, weighing 23 g. (normal, 4-7 g.), was removed, as also the phallus.

TABLE III

Daily hormone excretion of two pseudo-hermaphrodite sisters
(cases 13 and 14)

Patient	Date	17-Ketosteroids (mg)	Pregnanediol (mg)	Œstrogen (I. U)	Gonadotropin (M U)
Case 13 (P 499, aged 21 yrs.)	2/5/44	35 (60/litre)	50	100	...
	22/5/44	45 (56/litre)	36	...	...
	6/6/44	74	73	...	A.Z. test negative
	27/6/44	Left adrenalectomy performed	...	...	...
	19/7/44	50	not done	...	...
	27/7/44	32	31·5	...	...
	11/9/44	45	45	...	...
	15/10/44	62	75	...	...
	2/1/45	58	93	...	<30
	11/3/45	67	57	...	16
	5/6/45	66 5	58	...	...
Case 14 (K 42, aged 16 yrs.)	2/1/45	55	46	...	<20
	11/3/45	71	41	17	7·5
	5/6/45	64	56	...	...

Sections of the adrenal stained by Vines's method gave a positive androgenic reaction in 25-30 per cent. of cells in the middle zone and rather less than this in the inner zone of the cortex. A fair proportion of the cells in the outer and inner zones, but not many in the middle zone, showed a bloated, hydropic appearance. A wedge cut out of one ovary contained numerous small follicles, some of them atretic, but there was no evidence of corpora lutea or corpora albicantia.

A month after operation the 17-ketosteroids and pregnanediol excretion had fallen from 74 to 32 mg., but at the end of four months they had almost returned to the pre-operative level, indicating that hypertrophy of the remaining adrenal had occurred. The patient underwent a course of œstrogen therapy between October and January and this coincided with a consistently high pregnanediol output. The gonadotropin excretion was ascertained, as it was thought that this might be high in primary amenorrhœa, since the condition resembles that in climacteric women in whom the absence of ovarian function is associated with high gonadotropin titres. However the gonadotropin output (16 M.U.) was that of a normal adult woman.

The infantile condition of the ovaries makes it likely that the œstrogen recovered was secreted by the adrenals. Kenyon *et al.* (1937) examined the urine of 16 women with the adreno-cortical syndrome and found in all of them a diminution of œstrogen excretion as

compared with the normal. According to Werner (1941) a normal adult woman excretes 3-10 rat units of œstrone in 24 hours (approximately 15-50 I.U.) except between days 9 and 18 in the cycle, when values are equal to 100-250 I.U. Case 13 was therefore excreting more than the average woman at the height of reproductivity.

On grounds of hormone output alone it is difficult to explain why menstruation occurred in case 1 and not in case 13. It is unlikely that œstrogen secretion would be maintained at a constant level, and one would have thought that a semblance of " œstrin-withdrawal bleeding " could have occurred. The infantile condition of the uterus shows that there was no response to endogenous hormone stimulation on the part of the endometrium. It might be argued that the œstrogen/progesterone ratio was so markedly in favour of progesterone —case 13 excreted three times as much as case 1—that the action of œstrogen was neutralised ; later, owing to temporary lowering of this ratio during a course of œstrogen therapy, menstruation occurred for 7 days. When the course terminated in March 1946 she was excreting 17-ketosteroids 75 mg., pregnanediol 70 mg., and gonadotropin 51 M.U. per 24 hours. A comparison with case 11 (in whom " menstruation " occurred with the aid of œstrogen) cannot be pursued too far, as not only is it not known whether she has an adrenal or an ovarian anomaly, but no pregnanediol was excreted.

From the fact that there was no hypertension in this patient it is evident that hypertension is not caused by adrenal cortical hyperactivity as such ; hypertension occurred in a patient (case 1) excreting amounts which were much lower for pregnanediol and slightly lower for 17-ketosteroids.

The entire absence of hirsutism in a patient with marked adrenocortical hyperplasia is exceedingly rare, though not uncommon in cases of adreno-cortical carcinoma. Wieth-Pederson (1931, quoted by Cushing, 1932) described a woman with hypertension, striæ, dim vision, marked obesity, cardiac enlargement, polydipsia and headaches but without hypertrichosis, who was found at autopsy to have an adrenal cortical tumour 12 × 6 cm., with metastases. Complete absence of sexual symptoms in a man aged 33 in whom a large adrenal cortical tumour was found at autopsy is recorded by Anderson (1930) ; the patient died of hypoglycæmia. Lawrence (1937-38) describes a somewhat similar case, whereas severe diabetes was the sole symptom observed by Sprague (quoted by Kepler and Keating, 1941) in a patient in whom the condition entirely cleared up after the removal of a " huge " adrenal cortical tumour. Others reporting cortical tumours unassociated with symptoms of virilism are Cahill *et al.* (1942) 5 cases, and Gross (1940) 2 cases (both children) ; in the patient described by Anderson *et al.* hirsutism was very slight.

Until laparotomy, considerable doubt was entertained as to the sex of case 13, especially as, under the heading of " Male pseudohermaphroditism ", Allen (1939, p. 194) says : " There are human

cases with pelvic testes, vasa deferentia and prostates combined with almost complete internal and external female genitalia : oviducts, uterus, vagina, clitoris (more or less enlarged), mammary glands, *unbroken voice, lack of beard, female proportions of the body and female mentality.** . . . Its massed occurrence in families explains why the only interpretations which were proposed so far are based purely on genetics ". Out of 20 cases of male pseudo-hermaphroditism collected by Young from the literature, facial hair is described in 8 only, and in only one of these was it marked ; in the others it was generally " slight ". Seven are definitely stated to have had no facial hair, yet 19 out of the 20 had a penis. The importance of histological examination of the gonads in doubtful cases is forcibly illustrated by Young in describing his case 5.

It *was not possible to conclude that the patient (case 13) was female on the basis of œstrogen excretion alone*, as two men presenting many of the features of Cushing's syndrome excreted quite as much. The conclusion could certainly not have been reversed had the œstrogen output been low, since as little as 4 rat units of œstrogen has been excreted by women with adrenal tumours (*e.g.* Young's case 20).

GENERAL DISCUSSION

Considerable difficulty exists in differentiating between a tumour of the adrenal cortex or virilising tumour of the ovary and adrenal cortical hyperplasia on the basis of 17-ketosteroids excretion. This is clearly shown in table IV (p. 284). A 17-ketosteroids output exceeding 70 mg./24 hrs. in an adult is generally regarded as diagnostic of a tumour ; Dr Patterson thinks that the upper margin may have to be placed at 100 mg. Yet on the other hand an androgen output only a little above normal is described by Kenyon *et al.* in a case *of adrenal carcinoma, and in 7 adults in table IV it was less than* 60 mg. In contrast, out of 6 patients with hyperplasia of the adrenal cortex and no tumour, 4 had a 17-ketosteroids output exceeding 70 mg., one of them exceeding 100 mg.

Nor is it possible to effect a differentiation on the ground of pregnanediol output. The large amount of pregnanediol excreted by the two pseudo-hermaphrodite sisters—exceeding 70 mg. on three occasions—made an adrenal tumour likely, apart from the high values for androgens. Yet pregnanediol was excreted by only 3 out of 5 patients with adrenal cortical tumours, namely P 280 and cases 1 and 2, but not by cases 3 and 5 in spite of high ketosteroids output ; the two doubtful tumour cases 8 and 10 also excreted no pregnanediol. It is thus evident that, in marked adrenal involvement, both hormones are not necessarily involved simultaneously and that a high androgen excretion can exist apart from a high progesterone secretion. A high pregnanediol output in the absence of a high androgen excretion has *not yet* been encountered in such a case, though in case 2 its association

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* The italics are mine.

TABLE IV

Excretion of 17-ketosteroids in patients with tumours or hyperplasia of the adrenal cortex or virilising tumours of the ovary

Sex and age (years)		17-ketosteroids (mg./24 hrs.)	Author
**Adrenal tumours**			
M	1	28	Patterson *et al.* (1942)
M	1¼	2·8	Engstrom *et al.* (1944)
F	1¼	3·0	„
F	3	160	Talbot *et al.* (1942)
F	3½	5·9	Case 2 herein
F	3½	170	Engstrom *et al.*
F	3½	176	Fraser *et al.* (1941)
F	4	320, 166	Case 3 herein
M	5	27	Patterson *et al.*
F	6	126-288	Crooke and Callow (1939)
F	10	325	Friedgood and Whidden (1939)
M	11	84	Case 5 herein
F	13	166	Talbot *et al.*
F	16	54·6	Engstrom *et al.*
M	17	49	Case 4 herein
F	21	240	Engstrom *et al.*
F	21	50-55	Case 1 herein
M	25	40-64	Crooke and Callow
F	25	215	Anderson *et al.* (1943)
F	26	25-107	Callow and Crooke (1944)
F	34	270	Patterson *et al.*
F	35	74	Fraser *et al.*
F	38	54	Callow and Crooke
F	40	126	Warren (1945)
F	41	76, 78	Callow and Crooke
F	42	690	Warren
F	45	857	Engstrom *et al.*
F	49	269	Warren
F	54	83	„
F	56	74	Talbot *et al.*
F	61	14·5, 20	Callow and Crooke
F	63	45·6	Engstrom *et al.*
F	Adult	74	Talbot *et al.*
F	„	170	Patterson *et al.*
**Virilising ovarian tumours**			
	12	116	Prof. Jeffcoate's case, investigated on eve of going to press
	16	54·6	Kepler *et al.* (1944)
	22	40*	Abarbanel and Falk (1942)
	31	158	Warren
**Adrenal cortical hyperplasia with pseudo-hermaphroditism**			
F	3	13·5	Engstrom *et al.*
F	5	37	„ „
F	16	64, 71	Case 14 herein
F	18	109, 125	Solomons, (1943)
F	19	75·2	Engstrom *et al.*
F	21	67, 74	Case 13 herein

* Per litre.

with doubtful values of 17-ketosteroids provided a valuable aid to diagnosis.

In selecting the cases for group II, the definition of pseudo-hermaphroditism given by Young has been borne in mind. He defines it as " a condition always congenital in origin in which an individual, while possessing the gonads of but one sex, has other genitalia and . . . secondary characters belonging to the opposite sex " (p. 99). "It occurs once in a thousand persons " (p. 48). "Hyperplasia of the adrenal has apparently played an important part in the development of female pseudo-hermaphroditism " (p. 112). In chapter 5 of his book he cites 17 examples from the literature and adds four of his own, in all of which the condition was associated with greatly enlarged adrenals. He considers them (p. 103) excellent examples of the adreno-genital syndrome beginning in fœtal life and resulting in a condition characterised always by failure of the ovaries to develop and to stimulate the normal formation of the external genitalia. In such cases, he states, it is likely that the masculinising influence of the adrenal was dominant about the twelfth week of intra-uterine life and that this influence persisted.

Broster and Vines discuss fully the masculinising influence of the adrenal, especially ante-natally. In the fœtal adrenal of the male a strong fuchsinophil reaction is normally present in the cortical cells from the 10th to the 18th week at least, but in the female fœtus a weaker reaction occurs and lasts only from the 11th to the 14th week. The " male phase " in the female fœtus is thus very brief. One has to consider the time of differentiation of the genital tract of the female in relation to the changes which occur in the adrenal cortex. In man the ovary does not become differentiated from the indifferent sex gland until the 7th or 8th week ; the utero-vaginal canal appears about the same time in both sexes and in the female continues to develop until separation into uterus and vagina becomes histologically possible between the 12th and 16th weeks. As both uterus and vagina are generally present in female pseudo-hermaphrodites (*vide* cases 13 and 14), it is improbable that masculinisation becomes a dominant process much before the 12th week of fœtal life. Where the uterus is absent masculinisation must have occurred about the 9th or 10th week, at which time the uterine canal in the male undergoes atrophy. If the " male phase " of the cortex, which appears in the female at the 12th week of life, is superimposed, and should it persist and be wholly uncontrolled, not only will the rudimentary form of uterus and vagina usually found in pseudo-hermaphrodites be established but the ovary might be expected to undergo a partial reversal to testicular type. It is evident that the degree of sex reversal apparent at birth must depend on the period of fœtal life at which masculinisation becomes sufficiently dominant to initiate structural change.

The way in which the masculinising influence of the adrenal is exerted is not clear, but an observation made by Broster and Vines

(p. 84) suggests that the action may not be directly on the developing reproductive system but on the pituitary. In both sexes, after the 20th week of development, negative or very slight fuchsinophil staining is present in the cortical cells of the fœtal adrenal. Two female fœtuses were exceptions to this; in one at 22 weeks and in another at term strong staining of the cortical cells was obtained. In the latter, in which an adrenal cortical rest was also found, there was definite hyperplasia of the pituitary in which both basophil and (especially) eosinophil cells took part. In the 22-week fœtus, though a definite anterior pituitary hyperplasia was present macroscopically, there was no definite evidence of undue differentiation of eosinophil cells. There was no abnormality of the genital tract in either case.

An alternative possibility is that the syndrome of pseudohermaphroditism of very early infancy, when not due to adrenal tumour or hypertrophy, may be the result of the effect of hormonal activity in the mother upon the fœtus during gestation. A condition having many of the features of the human syndrome was induced by the author (Hain, 1935 a, b, c; 1936-37) in all the female offspring of rats injected during pregnancy with œstrogen or androgen. It was not ascertained whether the effect of the hormones was direct or via the adrenals. In the author's opinion an ante-natal excess of œstrogen is more likely to result in "constitutional" precocity of the type described by Novak (1944), of which the author has encountered three or four examples, than to give rise to pseudohermaphroditism. However, it is possible that the time at which the hormonal influence is brought to bear on fœtal development, and its amount, may profoundly modify the effect produced and thus a diversity of sex anomalies result.

According to Frazer (1931) the differentiation of the cortical region of the adrenal into its three zones is a late development which is continued after birth; the post-natal changes are imperfectly known. About the time of birth the cortex shows further differentiation: its greater part—known as the fœtal cortex—is covered superficially by a thin zone which is to become the adult cortex. This begins to grow after birth, while the fœtal cortex degenerates. The degeneration can be found in progress during the last two months of fœtal life or even earlier.

It might be thought, therefore, that the condition of the adrenal cortex bears a definite relation to the type and severity of the symptoms in virilism; but adrenal hyperplasia is a relatively common finding apart altogether from the adreno-genital syndrome, and so we must assume that in certain cases there is a functional as well as a structural disturbance. Moreover all adrenal neoplasms do not give rise to sexual changes; as a general rule these are most marked in malignant cases. Other forms of the adreno-genital syndrome are referable simply to overproduction of adrenal androgens, with the primary

stimulus residing either in the glands themselves or in the pituitary. There may be altered glandular function resulting in virilism without naked-eye changes in the adrenals or any increase in their size. Of 34 adrenals removed by Broster on account of virilism (Broster *et al.*, 1938, p. 164) 52 per cent. fell within normal limits, namely between 4 and 7 g., and 12 per cent. below this range ; all of them showed fuchsinophilia. Fuchsinophil cells were most abundant in the adrenal cortex of patients in whom the symptoms of virilism were associated with either tumour or hyperplasia of the cortex.

The intensity of the reaction seems to bear a definite relation to the degree of virilism. Cahill *et al.* state that the reaction is given by all adrenal cortical tumours, whether virilising symptoms are present or not, but is more pronounced when such symptoms exist. In the feminising tumour described by Simpson and Joll (1938) there were no discrete siderophil or fuchsinophil cells such as characterise virilising adrenal tumours, only diffuse fuchsinophil staining ; and both Broster and Vines (p. 78) and Goormaghtigh failed to find them in carcinomata without virilising symptoms or in " indifferent " adenomata. Vines observes that the reaction is not confined to the adrenal cortex, the stain being taken up also by the interstitial cells of the testis (p. 69), the cells of young corpora lutea (to both of which the adrenal cortex is cytologically related), and the acidophil cells of the pituitary. In cases of virilism, whether hyperplasia exists or not, the fuchsinophilia is most pronounced in the reticular zone, less so in the fasciculate zone and least in the zona glomerulosa. Since the masculinising function of the cortex is a physiologically normal process, which in virilism is exercised to excess, it is not surprising that fuchsinophil material is present in small amount in the adrenals of normal individuals. Sudds (1940) found the characteristic granules in the adrenals of 24 per cent. of adult males and 28 per cent. of adult females in a series which he examined, the percentage of granules increasing with age. They were not found in either sex before the age of 24 but were abundant in a 16-weeks-old fœtus.

If a definite relationship exists between the intensity of the reaction and the degree of virilism (as Vines believes), and assuming that the former is due to the androgen secreted, one would expect the excretion of 17-ketosteroids to be equally definitely related to the degree of virilism. This is far from being the case. Marked hypertrichosis may be associated with normal or even low values for 17-ketosteroids, whereas an output running into hundreds of milligrams may occur in another patient with hardly any virilism who has an adrenal cortical tumour or, more rarely, cortical hyperplasia.

An aid to differential diagnosis as between male and female pseudo-hermaphroditism presents itself in the fact that in only 0·7 per cent. of male pseudo-hermaphrodites is there persistent accessory adrenal tissue or bilateral hyperplasia of the adrenals. This was revealed in a survey of 970 case reports of pseudo-

hermaphroditism made by Neugebauer (1908), who also found that those bearing the male gonad are about 7 times more frequent than those with the female.' A normal 17-ketosteroids output such as that in cases 11 and 12 would be strong evidence in favour of regarding the patient as a male.

In discussing his case of male pseudo-hermaphroditism, Novak (1935) states that there have been many such cases reported "in which the only gonads possessed by the patient have been testes and in which, nevertheless, the external sex characteristics of the patients have been typically feminine. After all, every intersex is primarily male or female and, in the case of the human being at least, practically always female. If this assumption is correct, such patients as the one I have described, represent . . . genetic females in which the sex reversal took place at early phases of development, with complete replacement of ovarian by testicular elements. Depending on the time and intensity of the sex switch, all sorts of intergrades may develop . . .". On the other hand, that these were really *genetic males* is strongly suggested by the sex distribution in such families, taken from the records of Bonnier and Moebius and quoted by Allen (p. 195), in which, in a ratio of 58 females to 55 males, 42 of the latter were pseudo-hermaphrodites.

It is clear from a survey of the cases covered by this report that hyperactivity of the androgenic function of the adrenal cortex both ante-natally and post-natally has a profound influence on the sex characters of both sexes, the tendency being a virilising one in which the female characters are suppressed and male characters accentuated. In cases in which *male characters are weakened, e.g. in male pseudo-hermaphrodites*, it is feasible to suppose that the androgenic function was either weak or late in being established. Such a supposition does not necessarily run counter to the hypothesis put forward by Kallmann *et al.* (1944) of a hereditary basis for the condition. An alternative put forward by Young is that the defect may be due to malfunction of the interstitial cells of the testis. He believes that a certain quality of the gonad is required to bring about full development of the external genitalia and that in male pseudo-hermaphrodites the necessary stimulus is not at hand, with consequent failure of the penis, prostate and seminal vesicles to form. Young's suggestion implies that the gonad secretes before birth, which is doubtful; indeed, all the evidence (admirably reviewed by Moore, 1944) is strongly against such an idea.

SUMMARY

1. Six cases of adrenal cortical tumour are described, four of them in children, of whom two were boys. Hormone analyses of the urine showed a high androgen (17-ketosteroids) output in all cases except one ; some excreted pregnanediol also.

2. Unusual features in an adult woman with an adrenal tumour are discussed at length, namely associated ovarian involvement, persistence of menstruation, excess of the eosinophil cells of the pituitary, the character and size of the tumour and the excretion of a substance other than pregnanediol.

3. Eight cases of pseudo-hermaphroditism have also been examined, in all of whom doubt as to the sex existed at birth. Two of the children under 4 had siblings with adrenal cortical tumours and two were brought up contrary to the sex of their gonads. One of these, a male pseudo-hermaphrodite, excreted normal values of 17-ketosteroids; the other, a female pseudo-hermaphrodite "menstruated" on hormone therapy in spite of a high 17-ketosteroids output. The hormone excretion of two female pseudo-hermaphrodites (sisters) was observed over a prolonged period and showed that little benefit was derived from unilateral adrenalectomy.

4. The extent to which the hormone output is related to the extent and form of the adrenal cortical abnormality and the degree to which it can aid in the differential diagnosis are discussed. Apparently no cytological difference is discernible between neoplastic or hyperplastic adrenals which secrete progesterone in addition to 17-ketosteroids and those which do not. There is overlapping of 17-ketosteroids values in tumours and hyperplasia.

5. The mechanism by which the adrenal cortex brings about sex alterations antenatally is discussed.

REFERENCES

ABARBANEL, A. R., AND FALK, H. C.   1942.   *Endocrinol.*, xxx, S1025.

ADAMS, C. E.   1905.   *Trans. Path. Soc. London* (1904-05), lvi, 208.

ALLEN, E.   1939.   Sex and internal secretions, 2nd ed., London, p. 194.

ANDERSON, H. B.   1930.   *Amer. J. Med. Sci.*, clxxx, 71.

ANDERSON, A. F., HAIN, A. M., AND PATTERSON, J.   1943.   This *Journal*, lv, 341.

BALDWIN, J. F.   1914.   *J. Amer. Med. Assoc.*, lxiii, 2286.

BIDDULPH, C., MEYER, R. K., AND GUMBRECK, L. G.   1940.   *Endocrinol.*, xxvi, 280.

BROSTER, L. R.   1934.   *Lancet*, i, 830.

BROSTER, L. R., ALLEN, C., *et al.*   1938.   The adrenal cortex and intersexuality, London, p. 137.

BROSTER, L. R., AND VINES, H. W. C.   1933.   The adrenal cortex, London, p. 57.

BULLOCH, W., AND SEQUEIRA, J. H.   1905.   *Trans. Path. Soc. London* (1904-05), lvi, 189.

BURROWS, H.	1939.	*J. Endocrinol.*, i, 417.
BUTLER, G. C., AND MARRIAN, G. F.	1938.	*J. Biol. Chem.*, cxxiv, 237.
CAHILL, G. F., MELICOW, M. M., AND DARBY, H. H.	1942.	*Surg. Gyn. Obst.*, lxxiv, 281.
CALLOW, N. H., CALLOW, R. K., AND EMMENS, C. W.	1938.	*Biochem. J.*, xxxii, 1312.
CALLOW, N. H., CALLOW, R. K., EMMENS, C. W., AND STROUD, S. W.	1939.	*J. Endocrinol.*, i, 76.
CALLOW, N. H., AND CROOKE, A. C.	1944.	*Lancet*, i, 464.
CECIL, H. L.	1933.	*J. Amer. Med. Assoc.*, c, 463.
CROOKE, A. C., AND CALLOW, R. K.	1939.	*Quart. J. Med.*, xxxii, 233.
CUSHING, H.	1932.	Papers relating to the pituitary body, hypothalamus and para-sympathetic nervous system, *Springfield, Illinois.*
CUSHING, H., AND DAVIDOFF, L. M.	1927.	Monogr. Rockefeller Inst. Med. Res., *New York*, no. 22, p. 109.
DORFMAN, R. I., SCHILLER, S., AND SEVRINGHAUS, E. L.	1945.	*Endocrinol.*, xxxvii, 262.
ENGSTROM, W. W., MASON, H. L., AND KEPLER, E. J.	1944.	*J. Clin. Endocrinol.*, iv, 152.
FEKETE, E., AND LITTLE, C. C.	1945.	*Cancer Res.*, v, 220.
FORDYCE, A. D., AND EVANS, W. H.	1928-29.	*Quart. J. Med.*, xxii, 557.
FRANK, R. T.	1933-34.	*Proc. Soc. Exp. Biol. and Med.*, xxxi, 1204.
„	1937.	*J. Amer. Med. Assoc.*, cix, 1121.
FRASER, I.	1939-40.	*Brit. J. Surg.*, xxvii, 521.
FRASER, R. W., FORBES, A. P., ALBRIGHT, F., SULKOWITCH, H., AND REIFENSTEIN, E. C., JR.	1941.	*J. Clin. Endocrinol.*, i, 234.
FRAZER, J. E.	1931.	A manual of embryology, *London.*
FRIEDGOOD, H. B., AND WHIDDEN, H. L.	1939.	*New Engl. J. Med.*, ccxx, 736.
GESCHICKTER, C. F.	1935.	*Amer. J. Cancer*, xxiii, 104.
GLYNN, E. E.	1911-12.	*Quart. J. Med.*, v, 157.
GOORMAGHTIGH, N.	1940.	*Amer. J. Cancer*, xxxviii, 32.
GORDON, M. B., AND BROWDER, E. J.	1927.	*Endocrinol.*, xi, 265.
GRAEF, I., BUNIM, J. J., AND ROTTINO, A.	1936.	*Arch. Int. Med.*, lvii, 1085.
GROSS, R. E.	1940.	*Amer. J. Dis. Child.*, lix, 579.
GUTHRIE, L., AND EMERY, W. d'E.	1907.	*Trans. Clin. Soc. London*, xl, 175.
HAIN, A. M.	1935a.	*Edinb. Med. J.*, xlii (Trans. Edinb. Obst. Soc., 1934-35), 101.
„	1935b.	*Quart. J. Exp. Physiol.*, xxv, 131.
„	1935c.	*Ibid.*, xxv, 303.
„	1936-37.	*Ibid.*, xxvi, 29.
„	1942-44.	*J. Endocrinol.*, iii, 10.
HAIN, A. M., AND ROBERTSON, E. M.	1939.	*Brit. Med. J.*, i, 1226.
HARWOOD, J.	1931-32.	*Proc. Roy. Soc. Med.*, xxv, 951.
HERLANT, M.	1939.	*C.R. Soc. biol.*, cxxxi, 1315 and 1318.

HOLL, G. . . . . 1930. *Dtsch. Z. Chir.*, ccxxvi, 277.

HUTCHISON, R. . . . . . . 1904. *Brit. J. Child. Dis.*, i, 258.

JACOBZINER, H., AND GORFINKEL, 1936. *Amer. J. Dis. Child.*, lii, 308.
A.

JEANNENEY, G., AND LE CALVEZ . 1938. *J. Méd. Bordeaux*, cxv, 289.

JOSEPHSON, B. . . . . . . 1936. *Acta med. Scand.*, xc, 385.

KALLMANN, F. J., SCHONFELD, 1944. *Amer. J. Ment. Def.*, xlviii, 203.
W. A., AND BARRERA, S. E.

KENYON, A. T., GALLAGHER, T. F., 1937. *J. Clin. Invest.*, xvi, 705.
PETERSON, D. H., DORFMAN,
R. I., AND KOCH, F. C.

KEPLER, E. J., DOCKERTY, M. B., 1944. *Amer. J. Obst. Gyn.*, xlvii, 43.
AND PRIESTLEY, J. T.

KEPLER, E. J., AND KEATING, F. R. 1941. *Arch. Int. Med.*, lxviii, 1010.

KEPLER, E. J., KENNEDY, R. L. J., 1934. *Proc. Staff Meet. Mayo Clin.*, ix,
DAVIS, A. C., WALTERS, W., 169.
AND WILDER, R. M.

KEPLER, E. J., WALTERS, W., AND 1938. *Ibid.*, xiii, 362.
DIXON, R. K.

KRABBE, K. H. . . . . . . 1921. *New York Med. J.*, cxiv, 4.

LAWRENCE, C. H. . . . . . 1937-38. *Ann. Int. Med.*, xi, 936.

LIGHTWOOD, R. . . . . . . 1932. *Arch. Dis. Childh.*, vii, 35.

LONG, H. W., AND GRAY, J. W. . 1924. *Med. J. and Rec.*, cxix, 38.

LUKENS, F. D. W., AND PALMER, 1940. *Endocrinol.*, xxvi, 941.
H. D.

McGAVACK, T. H. . . . . . 1940. *Ibid.*, xxvi, 396.

McLETCHIE, N. G. B., AND SCOTT, 1942-44. *J. Endocrinol.*, iii, 347.
L. D. W.

MACERA, J. M. . . . . . . 1929. *Semana Méd.*, ii, 1481.

MAINZER, F. . . . . 1935-36. *Acta med. Scand.*, lxxxvii, 50.

MASON, H. L., AND KEPLER, E. J. 1945. *J. Biol. Chem.*, clxi, 235.

MATHIAS, E. . . . . 1922. *Arch. path. Anat.*, ccxxxvi, 446.

MECHLER, E. A., AND BLACK, W.C. 1943. *Amer. J. Path.*, xix, 633.

MILLER, I. D., AND KENNY, P. J. 1939-40. *Brit. J. Surg.*, xxvii, 728.

MOORE, C. R. . . . . . . 1944. *Amer. Nat.*, lxxviii, 97.

NEUGEBAUER, F. L. . . . . 1908. Hermaphroditismus beim Men-
schen, *Leipzig*.

NOBÉCOURT, P. . . . . . . 1934. *Progrès méd.*, lxi, 797 and 882.

NORRIS, E. H. . . . . . . 1938. *Amer. J. Cancer*, xxxii, 1.

NOVAK, E. . . . . 1935. *J. Amer. Med. Assoc.*, cv, 413.

„   . . . . 1944. *Amer. J. Obst. Gyn.*, xlvii, 20.

NUZUM, F R., AND DALTON, J. W. 1938. *Amer. Heart J.*, xvi, 643.

OPPENHEIMER, B. S., AND FISH- 1924. *Arch. Int. Med.*, xxxiv, 631.
BERG, A. M.

PATTERSON, J., McPHEE, I. M., 1942. *Brit. Med. J.*, i, 35.
AND GREENWOOD, A. W.

PLAYER, L. P., AND LISSER, H. . 1933. *Urol. and Cut. Rev.*, xxxvii, 758.

RHODES, A. . . . . 1943. *Arch. Ped.*, lx, 529.

ROWNTREE, L. G., AND BALL, R. G. 1933. *Endocrinol.*, xvii, 263.

SALMON, U. J., GEIST, S. H., AND 1941. *Proc. Soc. Exp. Biol. and Med.*,
SALMON, A. A.   xlvii, 279.

SCOTT, L. D. . . . . 1940. *Brit. J. Exp. Path.*, xxi, 320.

SHEPARDSON, H. C., AND SHAPIRO, 1939. *Endocrinol.*, xxiv, 237.
E.

SIMPSON, S. L., DE FREMERY, P., 1936. *Ibid.*, xx, 363.
AND MACBETH, A.

SIMPSON, S. L., AND JOLL, C. A. . 1938. *Endocrinol.*, xxii, 595.

SOLOMONS, E. . . . . . . 1943. *J. Obst. Gyn. Brit. Emp.*, 1, 363.

STRICKLER, H. S., SHAFFER, C. B., 1943. *J. Biol. Chem.*, cxlviii, 251.
WILSON, D. A., AND STRICKLER,
E. W.

SUDDS, M. V. N. . . . . . 1940. *Endocrinol.*, xxvi, 895.

TALBOT, N. B., BUTLER, A. M., 1942. *J. Clin. Invest.*, xxi, 559.
AND BERMAN, R. A.

TUFFIER, T. . . . . . . 1914. *Bull. Acad. de Méd.*, lxxi, 726.

VENNING, E. H. . . . . . 1938. *J. Biol. Chem.*, cxxvi, 595.

VENNING, E. H., WEIL, P. G., AND 1939. *Ibid.*, cxxviii, Sci. Proc., p. cvii.
BROWNE, J. S. L.

VON KUP, J. . . . . . . 1937-38. *Beitr. path. Anat.*, c, 137.

WALTERS, W., AND KEPLER, E. J. 1938. *Ann. Surg.*, cvii, 881.

WARREN, F. L. . . . . . . 1945. *Cancer Res.*, v, 49.

WEINBERGER, L. M., AND GRANT, 1941. *Arch. Int. Med.*, lxvii, 762.
F. C.

WERNER, S. C. . . . . . . 1941. *J. Clin. Invest.*, xx, 21.

,, ... 1943. *Ibid.*, xxii, 395.

WILKINS, L., FLEISCHMANN, W., 1940. *Endocrinol.*, xxvi, 385.
AND HOWARD, J. E.

WILSON, L., AND KURZROK, R. . 1940. *Ibid.*, xxvi, 955.

WOLFE, J. K., FIESER, L. F., AND 1941. *J. Amer. Chem. Soc.*, lxiii, 582.
FRIEDGOOD, H. B.

WOOLLEY, G. W., AND LITTLE, 1945. *Cancer Res.*, v, 203.
C. C.

YOUNG, H. H. . . . . . . 1937. Genital abnormalities, hermaphroditism and related adrenal diseases, *London.*

LIESEGANG RINGS AND ANTISEPTICS

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(PLATES XXXII AND XXXIII)

WHEN penicillin is titrated on agar plates by any of the now familiar "inhibition-zone" methods, concentric rings are often observed around the inhibition zone. In the course of experiments on blood-agar shake cultures, these concentric rings were formed in great variety and complexity. Before describing these rings some general considerations affecting periodic phenomena in bacterial cultures will be discussed.

TYPES OF RINGS

The term "inhibition zone" is misleading. If the surface of an agar plate is seeded and any anti-bacterial agent is diffused from a central focus the course of events may be studied by making coverslip impressions. It is found that many organisms including staphylococci multiply almost to the edge of the diffusion focus for about two hours; they then rather suddenly lyse over a certain area. A thin scum which often remains to indicate where growth has taken place produces one type of ring. This type is much better seen in chicken fibrin cultures. When staphylococci are added to chicken plasma and the mixture is clotted with embryo extract, the edge of an inhibition zone produced by penicillin shows a dense white halo which appears and disappears with changes of vapour pressure; this halo appears to occupy the position of lysed organisms.

A second type of ring appears only in shake cultures. Since the inhibition zone in such cultures is a section of a sphere, the deeper colonies in shake cultures are nearer the centre than surface colonies and produce a ring effect. The most striking rings, however, appear at the edge of the inhibition zone, where growth apparently begins. It is frequently found that the free edge of the growth is demarcated from the confluent growth by a ring of much diminished growth, and several concentric rings are sometimes found.

This appearance is more marked in shake cultures, especially when an anti-bacterial agent is floated on to the surface of shake cultures in chicken fibrin. If sections are cut, it is found that the colonies

SIMPSON, S. L., AND JOLL, C. A. . . 1938.   *Endocrinol.*, xxii, 595.
SOLOMONS, E. . . . . 1943.   *J. Obst. Gyn. Brit. Emp.*, 1, 363.
STRICKLER, H. S., SHAFFER, C. B.,   1943.   *J. Biol. Chem.*, cxlviii, 251.
    WILSON, D. A., AND STRICKLER,
    E. W.
SUDDS, M. V. N. . . . . . . 1940.   *Endocrinol.*, xxvi, 895.
TALBOT, N. B., BUTLER, A. M.,   1942.   *J. Clin. Invest.*, xxi, 559.
    AND BERMAN, R. A.
TUFFIER, T. . . . . 1914.   *Bull. Acad. de Méd.*, lxxi, 726.
VENNING, E. H. . . . . . . 1938.   *J. Biol. Chem.*, cxxxvi, 595.
VENNING, E. H., WEIL, P. G., AND   1939.   *Ibid.*, cxxviii, Sci. Proc., p. cvii.
    BROWNE, J. S. L.
VON KUP, J. . . . . 1937-38. *Beitr. path. Anat.*, c, 137.
WALTERS, W., AND KEPLER, E. J.   1938.   *Ann. Surg.*, cvii, 881.
WARREN, F. L. . . . . . . 1945.   *Cancer Res.*, v, 49.
WEINBERGER, L. M., AND GRANT,   1941.   *Arch. Int. Med.*, lxvii, 762.
    F. C.
WERNER, S. C. . . . . . . 1941.   *J. Clin. Invest.*, xx, 21.
    „   . . . . 1943.   *Ibid.*, xxii, 395.
WILKINS, L., FLEISCHMANN, W.,   1940.   *Endocrinol.*, xxvi, 385.
    AND HOWARD, J. E.
WILSON, L., AND KURZROK, R. .   1940.   *Ibid.*, xxvi, 955.
WOLFE, J. K., FIESER, L. F., AND   1941.   *J. Amer. Chem. Soc.*, lxiii, 582.
    FRIEDGOOD, H. B.
WOOLLEY, G. W., AND LITTLE,   1945.   *Cancer Res.*, v, 203.
    C. C.
YOUNG, H. H. . . . . 1937.   Genital abnormalities, hermaph-
                                              roditism and related adrenal
                                              diseases, *London*.

to be some action of methylene blue well outside the limits of its observed diffusion, since, if it is diffused into a blood-agar plate, hæmolysis is found after 48 hours outside the sharply defined blue zones. Again, if the products of bacterial metabolism react with blood pigments, a great variety of Liesegang effects is to be expected and is actually found. Finally, if the products of metabolism are acid, the inclusion of litmus in solution will bring to light further periodic phenomena.

It is now necessary to consider the conditions obtaining at any one point peripheral to a diffusion focus. It is assumed that the volume of the diffusing agent (as in the experiments to be described) is limited, and for the present purpose we shall neglect the fixation of diffusing agents discussed above.

FACTORS AFFECTING RING FORMATION

In a typical experiment shake cultures are made and when the gel sets holes are punched and filled with a solution of the anti-bacterial agent. The cultures are then incubated and the anti-bacterial agent begins to diffuse. But as it diffuses the organisms are growing. Two aspects of these processes deserve consideration. Firstly, the diffusing agent passes as a wave over any point, having initially a concentration of nil, rapidly rising to maximum concentration, and eventually falling and reaching stable and uniform concentration as it is diluted by the whole volume of the gel. Secondly, as the agent diffuses the organisms are growing. Hence the agent may reach the central organisms during their lag phase of growth, those more peripherally situated during their logarithmic phase, and the outermost organisms during their maximum phase. It is generally agreed that the sensitivity of organisms to anti-bacterial agents is related to their growth phase, and it is easy to understand how periodic phenomena may be produced by factors which alter growth rates in any area.

The diffusion of simple substances in liquids—particularly water—has been extensively studied and is well understood : temperature and concentration affect the diffusion of non-ionised substances, and ionised substances produce electric forces which retard their diffusion. The presence of a second ionised substance in the liquid alters the electric forces and therefore the rate of diffusion of the original substance. Diffusion in gels will be influenced by all these factors as well as by the gelling substance used, its concentration and its age. The gel is often capable of adsorbing the solvent in which the diffusing substance is dissolved.

The process becomes very much more complex when one substance diffuses into a gel containing another substance with which the first reacts, *i.e.* the Liesegang ring effect. This effect has been studied by many workers since Liesegang's original paper in 1896, but because

of the great number of variables involved the early work showed many contradictory results. The more important factors influencing these results would appear to be as follows.

The gel itself can have a great effect on ring production. Effects produced in gelatin are not necessarily repeated in agar and vice versa ; thus Hatschek (1919) found that silver dichromate formed bands in gelatin but not in agar, that lead chromate formed bands in agar but not in gelatin and that neither produced bands in a silicic-acid gel. Although this might have been expected, it was surprising to find that different brands of agar gave different effects, some even producing no rings at all (Hatschek, 1921). Müller (1916) was so impressed by the specificity of gels that he attributed the whole phenomenon to periodic structures in the gels themselves. Hedges and Myers (1926) state that another factor is the ageing of the gel, while the gel's previous history also appears to be important.

Light affects the formation of rings. Küster (1913) found that periodic lighting produced periodicity, but other observers produced similar effects in the dark. More recently Taboury and Bellot (1937) found that in the case they were investigating exposure to a bright light for a short time favoured the subsequent formation of rings in the dark, while prolonged exposure inhibited ring formation. Maxia (1939) quotes instances in which ultra-violet light influenced the rings obtained.

Other factors are the concentration of the gel itself and the concentration of the reactants. Stansfield (1917) concluded that periodicity was more probable if a strong diffusing agent and a dilute reactant were used, a conclusion supported in part by Morávek (1929).

However, the most troublesome factor to control is the presence of impurities. Even in small quantities these may have considerable effects : some produce and others inhibit periodicity ; some increase and others decrease the distance between rings ; and ethyl acetate may produce spirals. Schleussner (1922, 1924) found intermediate rings which he attributed to chlorides and phosphates originally present in the gelatin. Foster (1919) found that if she made up her gel with tap water instead of distilled water the amount of chlorides so introduced caused the silver dichromate rings to be formed in groups of three instead of singly. Now that most of the factors influencing results are known and attempts may be made to control them, it has become possible in recent years to study with greater certainty the effects of certain impurities in specific cases, and recent literature describes the effects of various acids, radicals and lipoids (Taboury and Echard, 1934 ; Taboury and Tournat, 1937 ; Taboury and Bernuchon, 1937). Recent theoretical treatment of Liesegang ring phenomena has been given by Neumann and Costeanu (1938) and Schemiakin and Mikhalev (1939).

It is apparent that the physico-chemical factors influencing the results in this type of experiment are many and varied. Because

Liesegang rings in bacterial shake cultures (litmus agar)
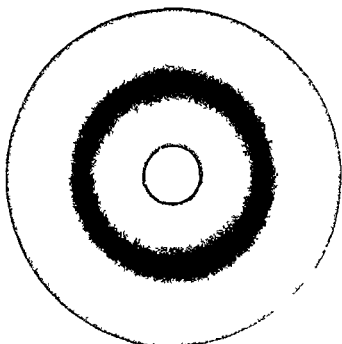
Fig 1 —*Bact coli*
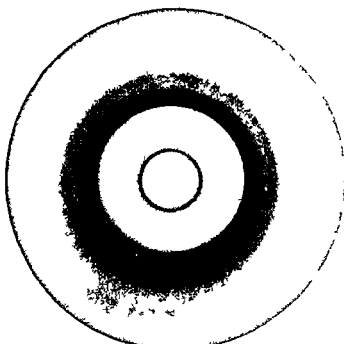
Fig 2 —*Bact typhosum*
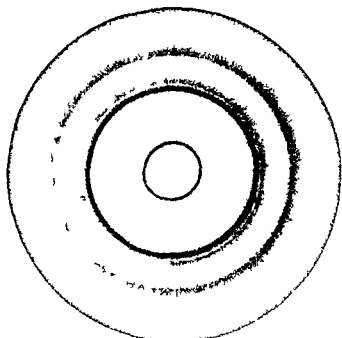
Fig 3 —*Bact paratyphosum* B
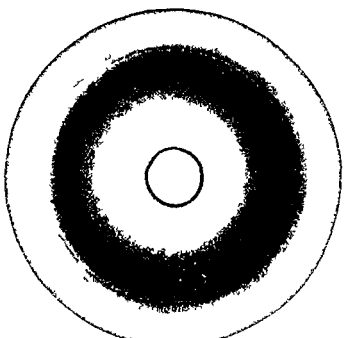
Fig 4 —*Bact cholerae suis.*
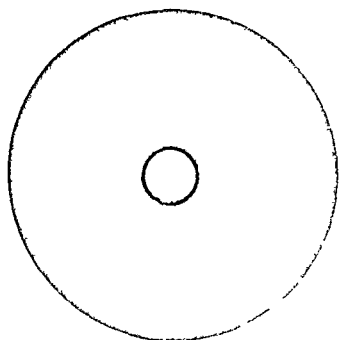
Fig 5 —*Bact enteritidis*
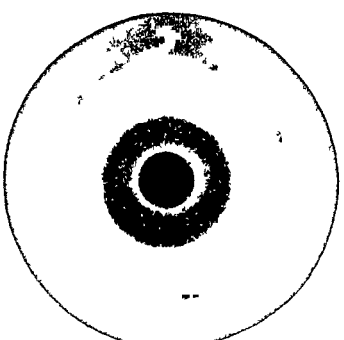
Fig 6 —*Bact* ...

LIESEGANG RINGS IN BACTERIAL SHAKE CULTURES (LITMUS AGAR)
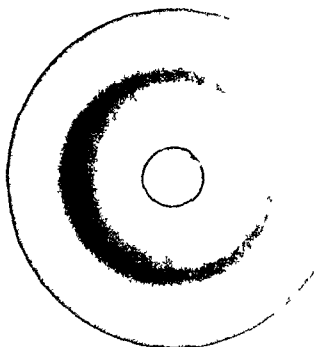
FIG 7—*Bact sonnei*
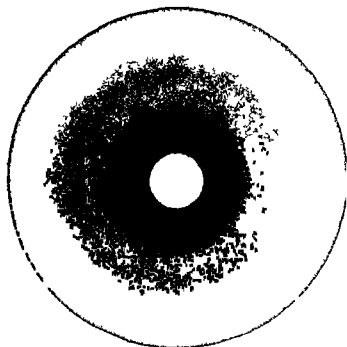
FIG 8—*Bact flexneri*
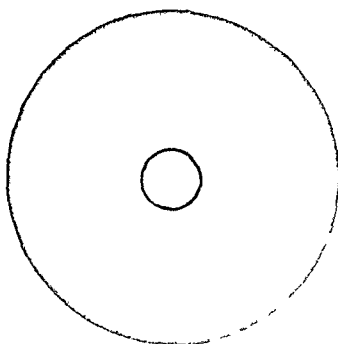
FIG 9—*Bact shigæ*
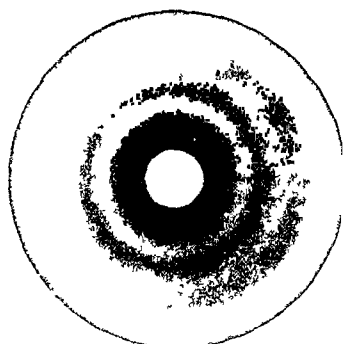
FIG 10—*Bact friedländeri*

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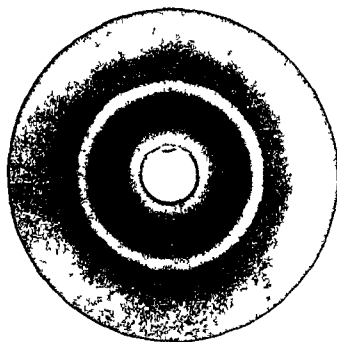
of this, because of the complex nature of the substances produced by bacterial metabolism and because of the effects of small quantities of foreign substances, it is obviously very difficult indeed to control conditions so rigorously that significant conclusions may be drawn from the ring patterns in various experiments. However, only some of the rings in the experiments described in this paper are due to a true Liesegang effect: the other ring patterns may well be of qualitative use for bacteriological purposes, but it must be borne in mind that other periodic phenomena are also very sensitive to the presence of small quantities of foreign substances (Hedges and Myers).

METHODS OF ACCENTUATING RING FORMATION

From these considerations it is to be expected that the periodic phenomena already observed on plate cultures with penicillin can be much exaggerated under appropriate conditions. In the remainder of this paper a suitable technique is given for their production and the results of its use are described.

Technique

1. Ten c.c. of Hartley's digest-broth agar are melted and placed in a 55° C. bath to cool. One c.c. of fresh oxalated human blood and 200 millions of the organism suspended in 1 c.c. of saline are added and thoroughly mixed. Plates are poured and when set are incubated for 1 hour at 37° C. Unless the blood is quite fresh and unless it is added to cooled agar, results are poor.

Agar plugs are then removed with a cork borer and the bottom is sealed with agar. The cup is then filled with the antiseptic to be tested, and the plates are incubated at 37° C. for 12 hours.

2. Alternatively 0·75 c.c. of litmus solution together with the organism is added to the melted agar. This technique was used, for the purpose of photography, with bismuth ammonium citrate in the experiments illustrated.

Results

When blood agar is used, a most intricate series of concentric coloured rings is found; these can be varied at will by any change in the conditions of the experiment. Some important variables are: (1) the species and type of organism; (2) its concentration; (3) the anti-bacterial agent; (4) its concentration; (5) the blood-pigment (e.g. oxy- or carboxyhæmoglobin); (6) the time elapsing between the inoculation of the agar and the application of the anti-bacterial agent; (7) the nature of the medium; and (8) the duration of incubation.

Up to thirty concentric rings have been counted and the colours are very vivid, including reds, greens and yellows. Since there are so many variables, only a few general comments will be made.

Organisms. Appearances when penicillin is used may be given as an example. Organisms produce substances which alter the

THE EFFECT OF TEMPERATURE ON IMMUNITY IN AMPHIBIA

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LITERATURE

MUCH of the early work on immunity in cold blooded vertebrates was intended to test their resistance to bacterial pathogens of man and other mammals Mesnil (1895), Ledingham (1922) and others tried unsuccessfully to infect fish and frogs with *Bacillus anthracis*, and agreed that their main defence was phagocytic Similar experiments have been many times repeated, and, since the pioneer work of Metschnikoff (1884, 1887), the importance of phagocytosis in bacterial disease of lower animals has usually been emphasised However, there is some evidence of the occurrence of humoral antibodies, both natural and acquired, in cold blooded vertebrates

Phisalix and Bertrand (1895) found anti venin in the blood of snakes, and Friedenthal (1900) demonstrated lysis of foreign blood cells by the sera of numerous lower vertebrates Tchistovitch (1899) found a hæmolysin active against many species in the blood of eels, and Kulp and Borden (1942) produced agglutinins in a bull frog by repeated injections of a bacterial vaccine

There is much evidence that infection in lower vertebrates is frequently caused by non specific organisms Wyss (1898), Babes and Riegler (1903) and Markoff and Jatschewa (1939) reported infection of fish with Proteus Nobécourt (1923) produced a fatal infection in frogs by a plant pathogen (*Bacillus caro tovorus*) Calmette (1922) and Williamson (1929) believed that infection of fish and frogs with saprophytic bacteria might occur Miura (1924) described cases in which phosphorescent bacteria derived from marine fish were pathogenic to freshwater fish Griffith (1941) found snakes susceptible to avian strains of *Mycobacterium tuberculosis* Harkins (1927) and others described the survival of *Erysipelothrix rhusiopathiæ* in fish Davis (1921 22) and Garnjobst (1945) described the infection of fish with a Myxococcus, normally harmless, but capable of the invasion of injured tissues at high water temperatures

In a previous paper (Bisset, 1946), I have demonstrated that fish may become infected naturally with saprophytic water bacteria, between whose invasive powers and the defences of the fish a balance appears to exist at low temperatures A rise in temperature to about 20° C will upset this balance, and the bacteria may then produce disease and even the death of the fish If the fish survives, however, it will clear itself completely of infection Fish appear also to have much greater resistance to initial infection at higher than at lower temperatures

In the bacterial disease of frogs known as "red leg", first studied by Sanarelli (1891) and Russell (1898) it was discovered by Emerson and Norris (1905) that, in frogs already infected with the disease, its course was completely controlled by the temperature and could be arrested by placing the frogs at a little above freezing point In a similar infection, Ernst (1890) had observed that frogs appeared to have a greater resistance to initial infection at a higher than at a lower temperature

These observations are entirely compatible with my previous findings in fish, and accordingly I decided to extend my investigations to amphibia.

EXPERIMENTAL OBSERVATIONS

The first experiments were attempts to raise the tadpoles of *Rana temporaria* in a sterile condition. It is quite simple to remove the ova from a female frog and fertilise them *in vitro* with an emulsion of the testis of a male ; but it is impossible to avoid contamination with bacteria, derived apparently from the oviduct, which gradually increase and spread through the albumin of the spawn.

In numerous unsuccessful attempts to sterilise the outside of the spawn with such substances as $KMnO_4$, mercurochrome, chloroform and so forth, the minimum concentration necessary to inhibit bacterial growth was invariably found to be greater than the maximum tolerated by the embryo. Penicillin was found ineffective because of the high proportion of Gram-negative bacteria present. It was also somewhat toxic to the embryo in high concentrations.

Eventually success was achieved by permitting the fertilised ova to absorb a solution of streptomycin (20 units per c.c.) in the process of the swelling of the albumin. Completely sterile embryos were raised up to the commencement of feeding. Because streptomycin is difficult to obtain this technique had to be discontinued, and it was therefore decided to employ a pigmented organism that was easily distinguishable from the bacteria occurring fortuitously in the tadpoles. Since the degree of infection of the peritoneal cavity was found to be small, and since fluorescent bacteria were seldom present, a strain of *Pseudomonas fluorescens* isolated from water was used for the remainder of the experiments. This organism was exceedingly tolerant of temperature variation, growing well at 37° C. and below 10° C., with an optimum in the region of 20° C. It rapidly produced a strong pigment and was thus readily detectable in impure cultures.

Inoculation of tadpoles

In the first experiments, 0·05 c.c. of a suspension made by emulsifying a few loopfuls of a 24-hour culture in 5 c.c. of saline was inoculated intraperitoneally into each of a large number of tadpoles. The death-rate from this inoculation was invariably high—from 30 to 70 per cent—and no significance has therefore been attached to it in the experiments on tadpoles. Conclusions have been drawn solely from the degree to which the bacteria were able to survive in the peritoneal cavity of inoculated specimens.

After inoculation the tadpoles were kept for 24 hours at room temperature, to which they had been accustomed, and the survivors were then placed in wide-mouthed 50 c.c. flasks—ten to a flask. Half of the flasks were kept at 8° C. and half at 20° C. The tadpoles

were fed on sterile minced meat, and at the end of a week they were killed by placing them in a solution of 5 per cent. urethane in water. They were then left for a few minutes in 1 per cent. mercuric chloride solution and opened with sterile precautions; cultures were made from the peritoneal fluid.

The results (table) show that the survival of bacteria is much greater at the lower than at the higher temperature.

TABLE

Survival of Ps. fluorescens *in tadpoles at 8° and 20° C.*

Batch	Temperature	Total no	No carrying *Ps. fluorescens*
1	8° C.	31	27
2	,,	20	17
3	,,	21	19
4	20° C.	18	3
5	,,	10	3
6	,,	20	14

There was no apparent difference in the health of the two groups.

Inoculation of adult frogs

For the subsequent experiments adult frogs were used. Two large frogs were kept at 8° C. and two at 20° C.: all were injected with 0·25 c.c. of a killed suspension of *Ps. fluorescens* intramuscularly in the thigh at intervals of two days. After 18 days they were killed and decapitated, and a few c.c. of blood were taken from each. Agglutination tests were performed against the homologous organism, which was agglutinated to a titre of 1 : 2000 by the serum of each of the two frogs immunised at the warmer temperature. The serum of the frogs immunised at the lower temperature gave no reaction at a titre of 1 : 80—the lowest dilution tested. The serum of two non-immunised frogs kept at 20° C. for a similar period gave no agglutinins.

Lastly, two strains of the same organism, which initially had a low pathogenicity for frogs, were passaged through two groups of frogs at 8° and 20° C. respectively. 0·25 c.c. of a suspension made by rubbing up a few colonies in 10 c.c. of saline was injected intraperitoneally. After two days a few drops of peritoneal fluid were aspirated with a syringe and cultivated on an agar plate at 20° C. As soon as sufficient growth had appeared, a few colonies were picked off and suspended in 10 c.c. of saline, and 0·25 c.c. was injected into the next frog. This technique was adopted instead of direct reinoculation of the peritoneal fluid in case any frog should be carrying other bacteria in its body cavity—as sometimes happened.

From the first six frogs at each temperature, cultures were made from aspirated fluid at 2-day intervals and it was discovered that the

degree of infection at 8° C. remained unchanged for at least two
weeks in every case. Some frogs kept at 20° C. became completely
sterile in four days; others did not completely clear themselves in
two weeks, although the degree of infection was greatly reduced.

During the 7th, 8th and 9th passages, the strain being passaged
at 8° C. was found to be losing its power of producing pigment.
Much as usual, this appeared after 18-20 hours' incubation, but it
never developed its full brilliance and faded almost completely after
3-4 days. To discover whether this was due to conditions of passage
or was an effect of temperature alone a strain of the original organism
was subcultured at 8° C. at 2-day intervals. After three weeks its
power of pigment production was unchanged.

After the 9th passage, the two strains of bacteria were inoculated
into two groups of six frogs, each of which received 1·5 c.c. of a very
heavy suspension intraperitoneally; all were kept at room temperature.
After 24 hours, all six of those which had received the strain passaged
at 20° C. (strain A) were dead. One only of those receiving the strain
passaged at 8° C. (strain B) was dead, but during the next day three
more died, though the other two survived. A similar dose was given
to two groups, each of twelve frogs. All that received strain A died;
two that received strain B survived. Most of those that received
strain A died much more rapidly than those that received strain B.

Two groups, each of nine frogs, were then given graded doses of
from 0·1 to 0·9 c.c. of a rather weaker suspension of each strain. In
24 hours, two that received strain A survived, one of which was
moribund; five that received strain B survived. Before passage,
the original strain was not strongly pathogenic and similar doses
had in all cases failed to produce death. These experiments indicate
that the increase in virulence caused by passage at 20° C. is much
greater than that caused at 8° C.

In the frogs dying of infection there was some slight evidence that
the invasiveness of strain A was also greater; because, in addition
to the greater speed of death, cultures taken from the muscles of
the thigh showed a more profuse growth of strain A than of strain B.
Cultures from the heart blood and from the peritoneal cavity showed
profuse growth in all cases.

Agglutinating antisera were prepared in three rabbits against
strains A and B and the original strain. Cross-agglutination reactions
showed that strain B and the original strain were antigenically
indistinguishable, but strain A did not agglutinate to so high a titre
as the homologous organism with antisera prepared against the other
two strains (1 : 500 for strain A and 1 : 1000 for the homologous
organisms).

DISCUSSION

From the above data it appears that the phenomena previously
recorded in fish (Bisset) may be general for cold-blooded vertebrates.

Bacteria and host, which are tolerant of one another at low temperatures, become intolerant when the temperature is raised.

To some extent, this fact is explained by the present demonstration that the production of circulating antibodies in the frog, which is both potent and rapid at 20° C., is completely inhibited at 8° C. None the less, the remaining defences of the frog appear to be sufficient at the lower temperature to restrain the invasiveness of the bacterium and to induce it to adopt a condition of balanced parasitism, although it remains potentially capable of producing disease and death. Paillot (1921) has made a similar observation in insects, but he claims that phagocytosis is also inhibited. The available evidence on this point is more fully discussed elsewhere (Bisset, 1947). At the higher temperature, the infecting bacterium appears also to become more readily exalted in virulence and correspondingly changed in antigenic structure, although it grows well at both temperatures.

SUMMARY AND CONCLUSIONS

1. Both adult and larval frogs rid themselves of bacterial infection more rapidly at 20° C. than at 8° C. At the lower temperature a condition of symptomless parasitism may result.

2. The production of agglutinins is inhibited at 8° C., whereas at 20° C. they are produced in high titre by inoculation with heat-killed vaccines.

3. Serial passage in frogs produces a much greater increase in virulence in a strain of *Pseudomonas fluorescens* at 20° C. than at 8° C.

4. A technique is described for the production of sterile amphibian embryos by the use of streptomycin.

REFERENCES

BABES, V., AND RIEGLER, P. . . 1903. *Cbl. Bakt.*, Abt. I, Orig., xxxiii, 438.
BISSET, K. A. . . . . . . 1946. This *Journal*, lviii, 251.
    ,, ... 1947. *J. Hyg.*, Camb., xlv, 128.
CALMETTE, A. . . . . . . 1922. L'infection bacillaire et la tuberculose chez l'homme et chez les animaux, 2nd ed., *Paris*.
DAVIS, H. S. . . . . . . 1921-22. *Bull. .U.S. Bureau of Fisheries*, xxxviii, 261.
EMERSON, H., AND NORRIS, C. . 1905. *J. Exp. Med.*, vii, 32.
ERNST, P. . . . . 1890. *Beitr. path. Anat.*, viii, 203.
FRIEDENTHAL, H. . . . . . 1900. *Arch. Anat. Physiol.*, Physiol. Abt., 494.
GARNJOBST, L. . . . . . . 1945. *J. Bact.*, xlix, 113.
GRIFFITH, A. S. . . . . . 1941. *J. Hyg.*, Camb., xli, 284.
HARKINS, M. J. . . . . . . 1927. *J. Bact.*, xiii, 32.
KULP, W. L., AND BORDEN, D. G. 1942. *J. Bact.*, xliv, 673.
LEDINGHAM, J. C. G. . . . . 1922. *Lancet*, ii, 898.
MARKOFF, W., AND JATSCHEWA, 1939. *Zbl. Bakt.*, Abt. II, c, 194.
Z. K.

MESNIL, M. A. . . . . . . 1895. *Ann. Inst. Pasteur*, ix, 301.
METSCHNIKOFF, E. . . . . . 1884. *Arch. path. Anat.*, xcvi, 177.
     ,, ... 1887. *Ann. Inst. Pasteur*, i, 321.
MIURA, H. . . . . 1924. Abstr. in *Jap. J. Med. Sci.*, i, 79.
NOBÉCOURT, P. . . . . . . 1923. *C.R. Soc. biol.*, lxxxviii, 1041.
PAILLOT, A. . . . . 1921. *Ibid.*, lxxxiv, 737.
PHISALIX, C., AND BERTRAND, G. 1895. *C.R. Acad. Sci.*, cxxi, 745.
RUSSELL, F. H. . . . . . . 1898. *J. Amer. Med. Assoc.*, xxx, 1442.
SANARELLI, G. . . . . . . 1891. *Cbl. Bakt.*, Abt. I, Orig., ix, 193 and
                                                 222.
TCHISTOVITCH, T. . . . . . 1899. *Ann. Inst. Pasteur*, xiii, 406.
WILLIAMSON, I. J. F. . . . . 1929. Fisheries Scot. Salmon Fish, II.
WYSS, O. . . . . 1898. *Z. Hyg.*, xxvii, 143.

INFLUENCE OF ŒSTRONE ON THE LYMPHOID TISSUES OF MALE MICE

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(Plates XXXIV-XXXVI)

A small number of mice of nearly all strains develop spontaneous lymphoid tumours   Cloudman (1941) gives a simple classification of the changes occurring in the lymphoid tissues of mice, in which he distinguishes three stages   hypertrophy of lymphoid tissues (hyperplastic nodules or lymphoma), neoplastic invasive growth of cells of fairly uniform type (lymphocytoma, leukæmic and aleukæmic), malignant growths of polymorphic cells (lymphosarcoma, leukæmic and aleukæmic)   Cole and Furth (1941) have shown that female mice of some strains exhibit a higher incidence of tumours of blood forming tissues than male mice   Hence it may be assumed that œstrogenic stimulation is associated with neoplasia of these tissues (Furth, 1946)   The following experiments were undertaken to investigate the influence of œstrone combined with castration on the lymphoid tissues of male mice of two laboratory stocks, designated H and R×H respectively   R×H stock was originated by crossing stock H with a stock R from another laboratory   These stocks are not inbred except in the general sense that animals kept in separate groups and allowed to breed freely are inbred   The stocks are maintained for the purpose of routine transplantation of standard tumours

Methods

Mice of H and R×H stocks were used in the present experiments   The incidence of lymphoid tissue changes in these two stocks is not high and may be less than 1 per cent

Male mice of each stock were divided into three groups , those of the first group were castrated and then painted with œstrone, those of the second were painted but not castrated, those of the third served as controls   Castration was performed on young mice of both stocks at the age of three or four weeks   The biweekly application of 0 01 per cent ketohydroxyœstrone in chloroform was usually started a week after the operation   Mice of H stock, both castrated and non castrated, were painted for six months , mice of the R×H stock were painted for four months   The thymus and lymph nodes were examined

microscopically at death; except when death occurred after only a few weeks of painting. All mice were maintained under similar conditions on rat-cake diet with an unlimited supply of tap water.

RESULTS

Several authors (Lacassagne, 1938; Gardner, Kirschbaum and Strong, 1940) have described the effect of ostrogens on mouse lymphoid tissue and have classified the changes induced into those affecting the lymphoid tissues and organs without involvement of the thymus gland, and those affecting the thymus, with or without involvement of other lymphoid tissues. The lymphoid tissue changes induced in the present investigations were divided into three groups: (1) those of the lymph nodes and other lymphoid organs without involvement of the thymus; (2) those affecting the thymus alone; (3) those of the thymus, lymph nodes and other lymphoid tissues and organs. The results of these experiments are presented in the accompanying table.

TABLE

Incidence of lymphoid tissue changes in male mice of two laboratory stocks

Stock	Experimental procedure	No. of mice	Average age at which response occurred (months)	Died before earliest response occurred	No response	Responded to treatment	Lymph-node changes, with changes in other lymphoid organs	Thymus changes only	Lymph-node and thymus changes, with changes in other lymphoid organs
H	Castration and painting	97	9·7	32	27	38 (59 per cent.)	22	7	9
	Painting	100	9·3	47	47	6 (11 per cent.)	4	1	1
R × H	Castration and painting	49	6·6	3	14	32 (70 per cent.)	10	12	10
	Painting	60	6·0	12	39	9 (19 per cent.)	1	3	5

It can be seen that œstrone painting alone caused lymphoid tissue changes in male mice of both stocks. The incidence of these changes was much higher in mice which were castrated as well as painted. This suggests that in both stocks elimination of the male sex hormone and excess of the female sex hormone result in stimulation of the growth of the lymphoid tissues. No changes occurred in the lymphoid tissues of 84 control mice (40 in H stock and 44 in R×H stock), although these lived for periods considerably longer than 20 months.

The difference between males of the H and R×H stocks lies in the rate of their response to œstrone treatment. The mice which responded to treatment showed signs of ill-health and emaciation. If the thymus was involved there was difficulty in breathing, with cyanosis. The lymphoid tissues exhibited changes which varied from simple enlargement to hyperplasia with invasion of the surrounding tissues.

PLATE XXXIV

INFLUENCE OF ŒSTRONE ON LYMPHOID TISSUE

Fig 1—Castrated mouse after 6 months treatment with œstrone showing enlargement of the thymus gland with invasion of surrounding tissues ×2

Fig 2—Castrated mouse after 4 months' treatment with œstrone showing enlargement of lymph nodes spleen and liver with involution of the thymus Slightly reduced
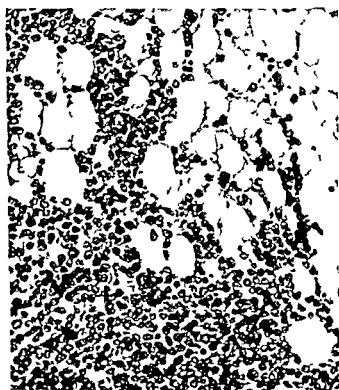
Fig 3—Lymphocytes of an inguinal lymph node penetrating into the surrounding adipose tissue Castrated mouse following 6 months' treatment with œstrone H and E ×250
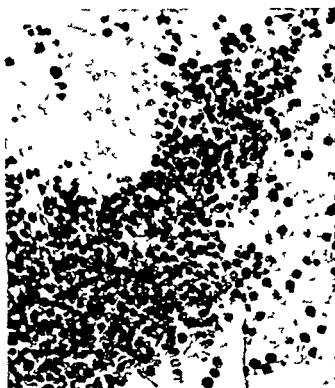
Fig 4—Lymphocytes of an axillary lymph node invading surrounding tissues Castrated mouse after 4 months' treatment with œstrone H and E ×250

Changes in the thymus gland

These comprised enlargement of the gland (fig. 1) and in most cases invasive growth into the lungs, trachea, diaphragm, pericardium, costal cartilages and intercostal muscles. In such cases a white mass of tissue filled the anterior mediastinum down to the diaphragm. The thymic changes were frequently accompanied by hypertrophy of the axillary, cervical, inguinal and mesenteric lymph nodes, and in addition the spleen, liver and kidneys were often enlarged.

Changes in lymph nodes

Enlargement of the axillary, inguinal and cervical lymph nodes was usually bilateral and the abdominal lymph nodes and spleen were often enlarged. In the majority of these animals the thymus was involuted (fig. 2), but in some it was enlarged and there was infiltration of the liver, lungs and kidneys by lymphoid tissue.

No changes were found on examination of blood from the tail or from the heart in any of the mice.

Histological changes

In the enlarged lymph nodes the normal structure was usually obliterated, the tissue consisting of fairly uniform large cells with numerous mitoses, while similar cells invaded the capsule and penetrated into the surrounding tissues (figs. 3 and 4). Similar conditions were found in relation to the enlarged thymus (figs. 5 and 6), where the enlarged lymphocytes had invaded lungs (fig. 7), pericardium (fig. 8), veins (fig. 9) and arteries (figs. 10 and 11), and had penetrated into the adjacent cartilages. Perivascular infiltration was most marked in the lungs and there was notable infiltration also of the kidneys (fig. 12).

This condition, characterised by overgrowth of uniformly enlarged lymphocytes with numerous mitoses and by their invasive growth into surrounding structures, resembles the lymphoid tissue change classified by Cloudman as lymphocytoma.

The earliest changes in males of the H stock appeared after three months, the latest after twelve months from the beginning of treatment. In the R×H stock the earliest changes were seen in six months and the latest in eleven months.

DISCUSSION

The influence of female sex hormone on the development of tumours and allied changes in the lymphoid tissues of mice is indicated by the higher incidence of changes in female mice of many strains (Cole and Furth). MacDowell (1936), however, found in C 57 mice that the

sexes were equally susceptible to the induction of neoplastic changes in lymphoid tissues. Mercier (1938) found lymphoid changes to be twice as frequent in female as in male mice. Lacassagne (1937, 1938) reported the production of lymphoid tumours after prolonged treatment with œstrogenic hormones. The majority of these tumours appeared in male mice of cancer-resistant strains and were lymphosarcomata of the thymus. Gardner, Kirschbaum and Strong (1940) increased the incidence of lymphoid tumours from 1 to 15 per cent., and Gardner (1942) from 2 to 25 per cent., by treatment with œstrogenic hormones. These tumours were successfully transplanted for many generations. Shimkin, Grady and Andervont (1941-42) reported the early development of lymphoid tumours after œstrogen treatment of strains of mice in which these tumours normally appear at an advanced age.

Some workers regard the part played by œstrogens in the induction of lymphoid tumours in mice as of greater importance than the genetic factor (Lacassagne, 1938). Others are of the opinion that the tendency to form lymphoid tumours after treatment with œstrogens is conditioned by the genetic factor, since some strains are more susceptible than others (Gardner, 1941 ; Gardner, Dougherty and Williams, 1944).

In view of the significance which has been attributed to the female sex hormone in the induction of lymphoid tissue changes in mice, experiments were carried out by several investigators to ascertain the influence of gonadectomy on the incidence of lymphoid tumours. In females of a strain of mice with a high incidence of neoplasia of blood-forming tissues, McEndy, Boon and Furth (1942-43, 1944) lowered the incidence by ovariectomy at the weaning age. Orchidectomy, however, had no significant influence on the incidence among males of the same strain. Gardner, Dougherty and Williams (1944), and Kirschbaum (1944) showed that gonadectomy did not significantly alter the incidence of neoplastic changes in mice of strains in which the incidence is approximately the same in both sexes. Murphy (1944) in another strain increased the male incidence by castration, but ovariectomy had no significant effect. Murphy considers the sex difference in susceptibility to spontaneous neoplastic changes of blood-forming tissues to be the result of an inhibitory effect by the male sex hormones. Miller and Pybus (1942) raised the incidence of lymphoid changes both by ovariectomy and by castration in the male. Thus experiments to ascertain the influence of gonadectomy on the development of lymphoid tumours have not yielded uniform results.

The present experiments suggest that the œstrogens exert a strong influence on the lymphoid tissues of male mice of the two laboratory stocks examined. Their administration induced lymphoid tissue changes and a greater increase of these changes followed orchidectomy combined with the application of œstrogens. While the average age at which the response occurred in castrated and

INFLUENCE OF ŒSTRONE ON LYMPHOID TISSUE
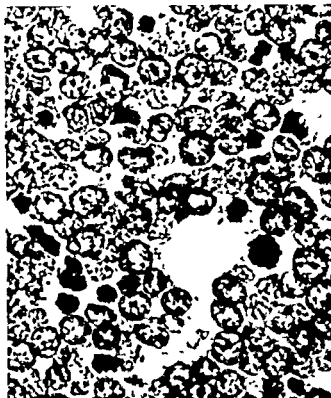
Fig. 5 —Cells of an enlarged thymus under-going mitotic division Castrated mouse following 4 months' œstrone treatment. H and E. ×350.

Fig. 6.—Cells of an enlarged thymus invading the intercostal muscles in a non castrated mouse following 6 months' œstrone treatment. H. and E. ×125.

Fig. 7 —Cells of an enlarged thymus invading the lungs in a non castrated mouse after 6 months' œstrone treatment. H. and E. ×100.

Fig. 8 —Cells of an enlarged thymus invading the pericardium in a castrated mouse after 6 months' œstrone treatment. H. and E. ×150.

PLATE XXXVI

INFLUENCE OF ŒSTRONE ON LYMPHOID TISSUE

FIG 9 —Invasion of a vein by cells of an enlarged thymus in a castrated mouse after 6 months' œstrone treatment. H. and E ×135.
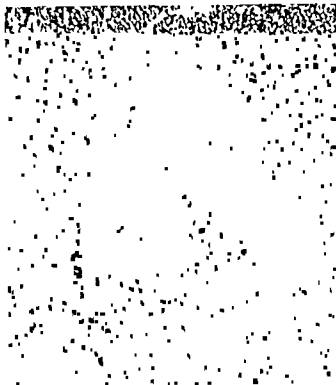
FIG. 10.—Invasion of an artery by cells of an enlarged thymus in a castrated mouse following 6 months' treatment with œstrone. H. and E. ×125.

FIG. 11.—The same under high magnification H. and E. ×200.

FIG 12.—Infiltration of the kidney from a local lymph node in a castrated mouse following 6 months' treatment with œstrone. H. and E. ×110.

non-castrated mice was approximately the same, it was lower in the R×H stock than in the H stock of mice.

Furth (1946) has pointed out that discontinuation of œstrogen treatment after three months induces a higher incidence of tumours of blood-forming tissues than continuous treatment, probably because of the longer life span of the mice. The high incidence of lymphoid changes obtained in the present experiments may be partially due to a somewhat similar curtailment of treatment (4-6 months).

It is difficult to decide whether the lymphoid changes in the present experiments (lymphocytoma of Cloudman) were of a truly malignant nature, as repeated attempts to transplant both the enlarged lymph nodes and the thymus were unsuccessful. The strains of mice were not homozygous, which may at least partially explain the failure of transplantation.

While in the present experiments some of the changes were induced during the application of œstrone, others appeared only 6-12 months after cessation of the treatment. It is not known whether this can be explained on the basis of Furth's suggestion that the mode of action of œstrogens is to induce atrophy of lymphoid tissue, which is followed by regeneration.

There are several factors at least which are able to increase the incidence of lymphoid tumours: heredity (Richter and MacDowell, 1935), X-rays and irradiation (Furth and Furth, 1936), carcinogens (Morton and Mider, 1938), hormones and nutrition (Furth, 1946). Gorer (1940) has suggested that " another factor, similar to the milk factor but transmitted in a different way, may play a decisive part in the incidence of other types of tumours ". The presence or absence of such a factor may account for the different results obtained by different workers in the induction of lymphoid tumours.

SUMMARY

The influence of œstrone on the lymphoid tissue of male mice of two laboratory (H and R×H) stocks of mice was investigated.

The mice were divided into three groups, of which one was painted with 0·01 per cent. ketohydroxyœstrone in chloroform, the second castrated and painted, while the third served as a control.

The changes in lymphoid tissue induced by the treatment consisted of local or general enlargement of lymph nodes or thymus gland or both, with or without invasion of surrounding tissues. Liver, kidneys and lungs were also frequently infiltrated but no blood changes were found.

Histologically these changes resembled neoplasia, but transplantation was unsuccessful.

The incidence of changes was as follows.

1. Painting alone: 11 per cent. in stock H; 19 per cent. in R×H stock.

2. Castration followed by painting : 59 per cent. in stock H ; 70 per cent. in R×H stock.

3. No changes appeared in any of the control mice.

The part played by œstrogens and other factors in the induction of these lymphoid tissue changes in mice is discussed.

REFERENCES

CLOUDMAN, A. M.	1941.	*In* Biology of the laboratory mouse, ed. by G. D. Snell, *Philadelphia*, pp. 213 and 214.
COLE, R. K., AND FURTH, J.	1941.	*Cancer Research*, i, 957.
FURTH, J.	1946.	*Physiol. Rev.*, xxvi, 47.
FURTH, J., AND FURTH, O. B.	1936.	*Amer. J. Cancer*, xxviii, 54.
GARDNER, W. U.	1941.	*Cancer Research*, i, 345.
,,	1942.	*Ibid.*, ii, 725.
GARDNER, W. U., DOUGHERTY, T. F., AND WILLIAMS, W. L.	1944.	*Ibid.*, iv, 73.
GARDNER, W. U., KIRSCHBAUM, A., AND STRONG, L. C.	1940.	*Arch. Path.*, xxix, 1.
GORER, P. A.	1940.	This *Journal*, l, 17.
KIRSCHBAUM, A.	1944.	*Proc. Soc. Exp. Biol. and Med.*, lv, 147.
LACASSAGNE, A.	1937.	*C.R. Soc. biol.*, cxxvi, 193.
,,	1938.	*Bull. Assoc. franç. Cancer*, xxvii, 96.
MACDOWELL, E. C.	1936.	*Amer. J. Cancer*, xxvi, 85.
MCENDY, D. P., BOON, MARY C., AND FURTH, J.	1942-43.	*J. Nat. Cancer Inst.*, iii, 227.
,, ,, ,, ,,	1944.	*Cancer Research*, iv, 377.
MERCIER, L.	1938.	*C.R. Soc. biol.*, cxxxvii, 92.
MILLER, E. W., AND PYBUS, F. C.	1942.	This *Journal*, liv, 155.
MORTON, J. J., AND MIDER, G. B.	1938.	*Science*, lxxxvii, 327.
MURPHY, J. B.,	1944.	*Cancer Research*, iv, 622.
RICHTER, N. M., AND MACDOWELL, E. C.	1935.	*Physiol. Rev.*, xv, 509.
SHIMKIN, M. B., GRADY, H. G., AND ANDERVONT, H. B.	1941-42.	*J. Nat. Cancer Inst.*, ii, 65.

SHORT ARTICLES

BACTEROIDES FUNDULIFORMIS (FUSIFORMIS NECROPHORUS)

A CHARLOTTE RUYS

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(PLATE XXXVII)

In the course of one year I received for determination three strains of obligate anaerobic Gram negative bacteria isolated from the blood of patients with a clinical picture of sepsis Dr B J Mansens, who sent me the first strain, provisionally labelled it as *B funduliformis* He cultivated the strain in August 1943 from a patient suffering from septic tonsillitis, twice from the blood and once from a pleural exudate After a very long illness the patient finally recovered

The second strain was isolated by Dr N Lubsen, working in my laboratory, in the spring of 1944, from the blood of a fatal case of sepsis probably due to a primary diverticulitis He twice obtained a pure culture in Tarozzi liver broth

Dr A J L Terwen cultivated the third strain from the blood of a patient suffering from a peritonsillar abscess and thrombophlebitis of the jugular vein followed by sepsis This patient also died

A fourth strain I cultivated myself in February 1945 from pus obtained from the knee joint of a patient with post partum pyæmia The pus also contained streptococci Special circumstances made it impossible to culture the blood of the patient in order to be certain whether death was due to streptococcal pyæmia or infection with the anaerobic micro organism

Strain I

The determination of the first strain gave rise to many difficulties Staining by Gram's method displayed unevenly coloured rods, often so pale that it was practically impossible to recognise them as bacteria In rather heavily grown brain broth media (Rosenow) often no rods could be seen, but many faintly coloured ball like shapes which did not show any structure whatever even with dark ground illumination Only after wet fixation in formaldehyde vapour and staining in Giemsa's solution was a very clear cut picture obtained, showing faintly blue protoplasm with brightly red stained particles

In the smears from the purulent pleural exudate this micro organism had the appearance of an evenly formed bipolar rod, but in culture media the picture was highly pleomorphic In some cultures unevenly stained rods dominated the field, in others there were long bluish threads with red granules at regular intervals, sometimes they seemed blown up like sausages (fundulus) The most curious picture was shown by the balls, which varied from very small to large conglomerates built up of bluish protoplasm and irregularly scattered red granules It was impossible to distinguish any regularity in the different stages in which the micro organism exhibited itself In some cultures small rods, long threads and small and large balls were seen in the same field The

balls varied from 2 to 12 μ in diameter ; the rods had a length of 2-3 μ and a width of 1/2-3/4 μ. Threads and sausages varied considerably in size (figs. 1-5).

Culturally, growth occurred only under anaerobic conditions and was not visible before 3-6 days. Rosenow's brain broth was the most satisfactory, liver broth with pieces of liver much less so. Shortly after isolation the colonies on glucose blood agar gave a faintly greenish tinge, which became much less pronounced in subcultures. Growth in several carbohydrate media was so variable that it was impossible to use it for classification. Subcultures in glucose broth or glucose agar lost viability. We cultivated the organism for 1½ years in the Rosenow medium and finally lost it in the spring of 1945 owing to the lack of gas and electricity in the laboratory. Immediately after isolation the strain was slightly pathogenic for guinea-pigs and mice. Inoculated subcutaneously it gave rise to a large infiltration in whose centre an abscess formed which broke open and healed after a week. A soluble toxin was not formed. Intraperitoneal inoculation of the strain was harmless. Subcultures lost their virulence very soon.

In accordance with the description of Weinberg *et al.* (1937) we labelled the strain provisionally as *Bacteroides funduliformis,* especially on account of its strange morphology. The only difference was that our strain did not grow readily in ordinary glucose broth. We succeeded in sending our strain to Paris and received the answer from Dr Reilly that he agreed with our identification.

The patient showed a clinical picture so well in accord with the descriptions in French literature (Teissier *et al.*, 1931 ; Lemierre *et al.*, 1940) that the physician, Mr Smit, had made the diagnosis of *funduliformis* sepsis even before the bacteriological examination which finally confirmed it.

Strain 2

This was also strictly anaerobic, but it grew better in Tarozzi's liver broth than in the Rosenow medium. It had less tendency to form balls and threads. These were numerous only in the first subcultures in brain broth. Later the rod form predominated and only occasional sausage-like forms or small ball shapes appeared. With Gram's stain they gave the same picture as strain 1, and only after wet fixation in formaldehyde vapour and staining with Giemsa's solution were the typical forms of figs. 6-10 seen.

Strain 2 was even less virulent for guinea-pigs and mice. A small infiltration at the site of inoculation disappeared in a few days. Because this strain also grew readily in glucose broth it is perhaps more in agreement with the description of *B. funduliformis* given by Weinberg than strain 1. In spite of the differences between the two strains I think it is justifiable to label them both provisionally as *B. funduliformis* (*Fusiformis necrophorus*), perhaps belonging to different types.

Strains 3 and 4

Strains 3 and 4 behaved in practically all respects like strain 2, except that in the first months there was very little pleomorphism. In the spring of 1945 strains 2 and 3 were lost, but with strain 4 I observed a few months later the typical sausage-like appearance and from time to time also the small balls. So in spite of the rarity of the special forms in the first months after isolation I am convinced that both strains belong to the same species as strain 2.

Strain 5

In February 1946 I received a strain of *B. funduliformis* isolated in 1940 by the late Prof. Kapsenberg at Groningen from the blood of a child suffering from sepsis after otitis media complicated with thrombosis of the lateral sinus and

BACTEROIDES FUNDULIFORMIS (FUSIFORMIS NECROPHORUS)
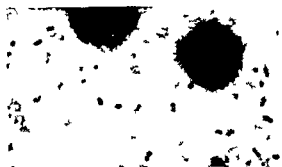
Fig 1
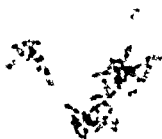
Fig 2

Fig 3

Fig 4

Fig 5
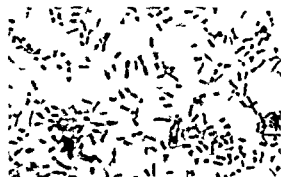
Fig 6
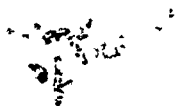
Fig 7

Fig 8

Fig 9

Fig 10

Bacteroides funduliformis (Fusiformis necrophorus)   Wet fixation in formaldehyde
vapour   Giemsa stain   ×1000

jugular vein   The strain was cultivated from the sinus and was mixed with staphylococci   Prof Kapsenberg succeeded in getting a pure culture which was kept alive for 6 years in Tocunaga agar   The patient recovered after a prolonged illness   In my hands the organism behaved much like strain 1, especially in morphology   It grew however much less readily in brain broth   This difference may have been due to the fact that it was cultivated for so many years in quite another medium

The future will show whether isolation of several strains of *B fundiliformis* during the war means that lowered resistance has favoured invasion by this micro organism   Because sulphonamides have not proved of much value in the treatment of these infections, I tried whether my own remaining strain and that of Prof Kapsenberg, of different types, were perhaps sensitive to penicillin

The penicillin was diluted in Tarozzi liver broth in such a way that each tube contained 10 c c of the medium with falling doses of penicillin   To each tube 5 drops of a well grown young culture were added ; in another experiment the tubes were much more heavily seeded   The results were read after 2 and 8 days   Check tests were made with penicillin alone and without penicillin   The concentration of penicillin was from 5 units to 0 1 unit per c c of the medium   Two different samples (a and b) were used   As the table shows, both types of

TABLE

Growth of B fundiliformis *in Tarozzi liver broth with the following concentrations of penicillin*

Strain no	Quantity of culture added	5 units per c c	1 unit per c c	0 5 unit per c c	0 1 unit per c c	0 unit per c c
		Penicillin (a) (C S C)				
4	5 drops	—	—	—	+	+
5	5 ,,	—	—	—	+	+
4	1 c c	—	—	+	+	+
5	1 ,,	—	—	+	+	+
		Penicillin (b) (Chas Pfizer and Co)				
4	5 drops	—	—	—	+	+
5	5 ,	—	—	—	+	+

B fundiliformis are sensitive to concentrations of penicillin which can be reached in the blood by normal treatment   As the disease often ends fatally it will be worth trying penicillin treatment in every case where *fundiliformis* infection is suspected   A short statement in the *Presse médicale* (16 Feb 1946, Bull Hop de Lyon) that a case of *fundiliformis* sepsis recovered after treatment with penicillin supports this view

Summary

Four strains of *Bacteroides fundiliformis* (*Fusiformis necrophorus*) were isolated from blood and pus of patients suffering from sepsis   Wet fixation in formaldehyde vapour and staining with Giemsa's solution showed red granules in a bluish protoplasm   In smears of pus the rod form prevailed ; in cultures, a highly pleomorphic picture was common   The strains belonged to two types, in one the ball and sausage like forms prevailed, in the other the rod form   Penicillin concentrations of 1 to 0 5 unit per c c inhibited the growth in Tarozzi liver broth of both types

REFERENCES

LEMIERRE, A., GUIMARAES, A. P.,   1940.   *Presse méd.*, xlviii, 97.
    AND LEMIERRE, J.

TEISSIER, P., REILLY, J., RIVA-   1931.   *Ann. de Méd.*, xxx, 97.
    LIER, E., STEFANESCO, V.

WEINBERG, M., NATIVELLE, R.,   1937.   Les microbes anaérobies, *Paris.*
    AND PRÉVOT, A. R.

576 . 851 . 49 (*Shigella flexneri*) : 576 . 8 . 093 . 3

NON-MANNITOL-FERMENTING *SHIGELLA FLEXNERI*, TYPE 103

M. G. NELSON

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Halton, and Queen's University, Belfast*

In the summer of 1945 seven strains were isolated of a Flexner-type dysentery organism with aberrant sugar reactions.   Six of the strains came from the stools of nine cases of bacillary dysentery occurring as a small localised outbreak in one R.A.F. mess at Takoradi, Gold Coast.   The seventh strain was found in the stool of one of the African mess boys employed in handling food at the same mess ; he was a healthy carrier and presumably the source of infection.   On MacConkey's medium and sodium desoxycholate (Hynes's modification), the strains appeared in the smooth phase on primary isolation. All were non-motile when tested by Craigie's (1931) tube method and all gave similar biochemical reactions (table).

TABLE

Biochemical reactions of aberrant dysentery strains

Motility	Indole	Glucose	Lactose	Mannitol	Sucrose	Maltose	Arabinose	Xylose	Dulcitol	Inositol	Salicin	Methyl red	Voges-Proskauer	Litmus milk	Urea PO₄	Gelatin
—	+	A	—	—	—	A	A	—	—	—	—	—	—	N.C.	—	—

A = acid ; N.C. = no change.

Serological reactions

The strains formed indole and fermented only glucose of the four primary sugar substrates used (glucose, lactose, mannitol and sucrose).   *This biochemical pattern suggested a diagnosis of Shigella schmitzi* but the strains failed to agglutinate with Schmitz antiserum.   They were then tested against sera prepared with Sachs's arabinose-fermenting para-Schmitz strain Q 902, but again with negative results.   However, when the organisms were tested against polyvalent Flexner 2 antiserum (Oxford Standards), significant agglutination resulted.   On primary isolation the strains agglutinated to about 1 : 100 with

polyvalent Flexner 2 antiserum, but on subculture all went to full titre at 1 : 250. On primary isolation the strains were specifically agglutinated in low titre by Oxford Standards Flexner 103 antiserum and failed to react with other Flexner type antisera. In subcultures all showed a marked tendency to rough variation associated with a rise in titre against Flexner 103 antiserum and a broadening of the antigenic range against other Flexner type antisera.

These findings suggest that Oxford Standards antisera against the Flexner group are low in type-specific agglutinins and high in group component, which may cause difficulty in diagnosis with recently isolated strains where the type-specific antigen is dominant. In diagnostic work monovalent absorbed antisera containing only the type-specific component have many advantages and allow rapid and specific typing of members of the Flexner group. In practice it is difficult to prepare a specific antiserum with a high enough diagnostic titre ; and a second difficulty is that such a specific absorbed antiserum determines only the presence of the type-specific antigen and not the complete antigenic structure. The sub-races VZ and WX of Andrewes and Inman (1919) contain the type-specific antigen of one type and the group antigenic structure of another. This diagnostic difficulty can be obviated and the complete antigenic analysis made by testing all strains which react with monovalent absorbed Flexner V and W antisera with other appropriate antisera to determine the group antigenic structure. The seven non-mannitol-fermenting strains were tested against a range of specific absorbed Flexner antisera and reacted only with Flexner 103. This proved that these strains possessed the Flexner 103 specific antigen. Their complete antigenic identity with Flexner 103 (Oxford Standards and N.C.T.C. strains) was proved by mirror-absorption tests, which demonstrated that the new strains possess both the specific and the group antigenic structure of *Sh. flexneri*, type 103. An antiserum prepared with one of the non-mannitol-fermenting strains specifically agglutinated the other six as well as four mannitol-fermenting Flexner 103 strains recently isolated from cases of bacillary dysentery and the N.C.T.C. Flexner 103 strain—all to the same titre.

Discussion

The finding of seven strains of an organism with the biochemical reactions of *Sh. schmitzi* but the antigenic structure of *Sh. flexneri*, type 103, again demonstrates the unreliability of the so-called basic biochemical reactions of the Flexner group. Confidence in these was first undermined by the discovery of the gas-producing Newcastle variant of *Sh. flexneri* by Clayton and Warren (1928-29, 1929-30), and later of the Manchester variant by Downie, Wade and Young (1933).

More recently Orr-Ewing and Taylor (1945) have isolated ten strains of an organism with the biochemical reactions of *Sh. shiga* and the antigenic structure of Flexner-Newcastle. These authors have stressed the importance of testing with Flexner-Newcastle antiserum all strains which give the biochemical reactions of Shiga but are not agglutinated by Shiga antiserum—a warning which could be logically extended to testing all " para-Shiga-Schmitz " strains with polyvalent Flexner antisera.

There have recently been numerous reports of the isolation of para-Shiga-Schmitz organisms from sporadic and epidemic cases of diarrhœa and dysentery. Organisms of this type have been broadly classified biochemically by Sachs (1943), who recognises many antigenic types of each biochemical variant. But before any organism is classified as a para-Shiga-Schmitz or Sachs type, great care is necessary to exclude *Sh. flexneri* (aberrant biochemical variant) and *Proteus morgani*. The use of polyvalent Flexner antisera will readily exclude the first, but organisms of the *P. morgani* type may cause more difficulty.

Many fæcal strains of *P. morgani* are poorly motile or non-motile, and may

616 . 682—006 . 45

TWO CASES OF ADENOFIBROMYOMA OF THE EPIDIDYMIS

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(PLATE XXXVIII)

In a previous paper, " Zur Kenntnis der primären Nebenhodengeschwülste " (Falconer, 1938-39), I described a case of adenofibromyoma in connection with a survey of the primary tumours of the epididymis. That case and one described by Sakaguchi (1916), mentioned in the same paper, seem to be the only ones published hitherto.

Case reports

Case 1. The patient was a 39-year-old man, operated on for acute suppurative appendicitis at the Karolinska Sjukhus. For about five months he had observed a hard lump on the right testicle. On examination, there was found, adjacent to the distal pole of the testicle, a hard, resistant nodule about the size of a hazel nut. At operation a firm, rounded, well-defined growth was removed from the cauda epididymidis. The parietal layer of the tunica vaginalis was partly adherent to the surface of the testicle but there were no signs of recent inflammation. The operative diagnosis was epididymitis (?).

Case 2. The patient, a 36-year-old man, was admitted to hospital (St Görans Sjukhus) because of a tumour in the scrotum, first observed 6 years before. It was about the size of a hazel nut and was extirpated along with the epididymis. The operative diagnosis was tumour (?), tubercle (?).

Histology

As the microscopic structure of these two tumours was practically identical, the following histological description applies to both. The main part of each tumour consists of smooth muscle (fig. 3) showing in places a plexiform arrangement, and of collagenous connective tissue. Between the bundles of muscle and connective tissue there are groups of epithelial cells (fig. 2), principally in the form of solid cords, though sometimes appearing as thin glandular tubes. The cells, which are fairly large, are sometimes cubical or cylindrical, but most frequently polygonal, with well-stained cytoplasm, their shape varying within one and the same cord or tube. Some of their nuclei are vesicular, with visible nucleoli, others are more compact. No cilia are seen, nor any signs of secretory activity. Interstitial foci of round cells are present in places. There are no features suggesting malignancy, and the pathological diagnosis is adenofibromyoma.

ADENOFIBROMYOMA OF EPIDIDYMIS

Fig 1 —Author's pre
viously reported case
Field showing a num
ber of faintly stained
epithelial cords above
and to the left and a
broad zone of inter
stitial infiltration by
round cells immediate
ly below Hæmatoxy
lin and van Gieson
× c 210

Fig 2 —The present
case 1 Field show
ing epithelial cords
mainly differentia
ted but with occa
sional lumina and
surrounding bundles
of unstriped muscle
Hæmatoxylin and
eosin × c 210
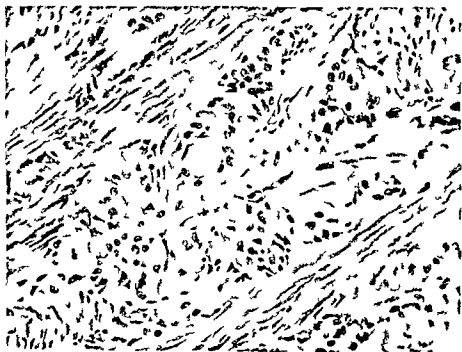
Fig 3 —The present case 2
Field showing a notable
preponderance of the fibro
muscular over the epithe
lial element Hæmatoxylin
and van Gieson × c 210

urinary bladder. The epithelium is poorly differentiated, the cells being large and well stained but of no definite shape (cylindrical and cubical as well as polygonal). The epithelium is mainly in the form of solid cords (fig. 2); lumen formation is less frequent. There are no cilia nor any signs of secretion. The tumour is thus of organoid type, and imitates an organ built up of epithelium-lined ducts and a fibro-muscular apparatus (fig. 3). Its nature as a malformation tumour on an embryonal basis is thus evident, and Sakaguchi, in analysing his case, arrived at the opinion that the tumour probably originates from remnants of the Wolffian duct.

The "matrix organ" of the tumour—the epididymis—develops, as is well known, from the Wolffian duct and the transverse ducts of the primordial kidney. At the upper and lateral part of the epididymis there is a small process, the appendix epididymidis, and several so-called ductuli aberrantes have also been described whose microscopical structure corresponds on the whole with that of the epididymis itself. An adenofibromyoma may be considered to show the same structure on an earlier, less differentiated plane. In agreement with Sakaguchi, I too thus find it most likely that the tumour in question has the same embryonal origin as the epididymis, being possibly derived from some aberrant part of this organ.

In this connection I should like to draw attention to the possibility that an adenofibromyoma increases in size under the influence of gonadotropic hormones in the same way as the rest of the sexual apparatus during puberty. This assumption is supported by the fact that three of the four reported cases occurred in patients in the sexually active period, their respective ages being 32, 39 and 36 years. As regards my first case—the one published in 1939—there are no data in the hospital journal to show how long the tumour had been in existence.

From the clinical point of view it is obviously impossible to differentiate a tumour with the above-described clinical characters from an old epididymitis.

Summary

Two new cases of adenofibromyoma of the epididymis are described and reference made to two previously published cases. The tumour, which is benign, is regarded as probably originating from the Wolffian duct and transverse ducts of the primordial kidney. It is suggested that its development may be hormonally determined.

REFERENCES

FALCONER, B. . . . . 1938-39. *Z. Krebsforsch.*, xlviii, 243.
SAKAGUCHI, Y. . . . . 1916. *Frankf. Z. Path.*, xviii, 379.

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TUBULAR ADENOMA OF THE TESTIS AND
ŒSTROGENIC ACTIVITY

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(PLATES XXXIX AND XL)

Tubular adenoma or Sertoli-celled tumour of the testis was first described by Pick (1905, 1916), who found it in ectopic testes and once in the ovary of a pseudo-hermaphrodite. Masson (1923) recorded the appearances of the tumour

in detail, while Krückmann (1936-37) in reviewing the literature found 16 cases in ectopic testes, 3 in the ovaries of women with menstrual disorders but no other recorded sexual troubles and 16 in the testes of *pseudo-hermaphrodite* males. Innes (1942) recorded 15 cases in the testes of dogs. In 5 of these the affected testis was ectopic, alopecia of the abdomen occurred in 3 and reversal of sexual attractiveness in 2. Profuse growth of hair followed removal of the tumour from the bald dogs. The tumours were all clinically benign and no changes in the other organs were recorded.

Case report

The subject was a pekingese dog aged four years. His left testis had never descended, but the right appeared to be normal. His coat was of the profuseness usual to this breed. Four months before death he was examined by a veterinary surgeon in the course of a routine visit to the owner's house. The right testis was found to be normal, but there was no trace of the left. Three months later, however, the veterinary surgeon was called in again and found the dog to have a tumour of the right testis which was said to have been growing in a uniform manner for about a month. The whole testis was evenly enlarged, there was no hydrocœle and no adhesion to the scrotum. Alopecia and pigmentation of the belly had appeared at the same time as the tumour and was increasing but although the result resembled the feminine habitus, the mammæ were not enlarged. No disturbance of sexual behaviour had been noted, but the dog was not known to have bred at any time. About two weeks later the tumour was removed. Four days after the operation, however, the dog developed retention of urine and was destroyed.

At necropsy within a few hours of death the left testis was found to be represented by a mass of fibrous tissue about one cm. in length just within the internal inguinal ring. The operation site was clean. The prostate was generally and much enlarged, forming a mass some 4 cm. in diameter, which filled the pelvis. Its capsule was expanded but not invaded, and the tumour appeared to be benign. The bladder was distended with urine: all the other organs appeared normal.

Detailed descriptions of the tumours

I. Testis. The size and shape of the tumour is well shown in the illustration (fig. 1). It is firm in consistency, with prominent fibrous trabeculæ, and pinkish grey in colour. The whole of the organ is involved, but the epididymis is normal and the tunica albuginea is not invaded. In some areas, sections show the cells to be arranged in a palisade manner, radiating out from the thickened and fibrous walls of the seminiferous tubules (fig. 2), their nuclei fusiform and with their long axes perpendicular to the tube walls. In other areas the cells, though of similar structure, are arranged in irregular masses separated by collagenous septa (fig. 3). These appearances are exactly those described by Masson and by Innes.

II. Prostate. The organ consists of a number of small cysts separated by fibrous-tissue trabeculæ. The microscopical structure, as revealed by six blocks from different areas chosen at random, is singularly uniform. The lining epithelium of the cysts shows hyperplasia and metaplasia, so that it becomes markedly stratified and eventually desquamates, forming prominent conglomerated masses in the cavities of the cysts (fig. 4). At the edge of the organ, these processes can be followed throughout their various stages.

Discussion

In nine enlarged prostates from dogs, Zuckerman and Groome (1937) found only one which corresponded with that from the present case, both in its large

TUBULAR ADENOMA OF TESTIS

FIG. 1.—Testicular tumour on section.   Natural size.

FIG. 2.—Testicular tumour under low power to show tubular architecture.   ×75.

TUBULAR ADENOMA OF TESTIS
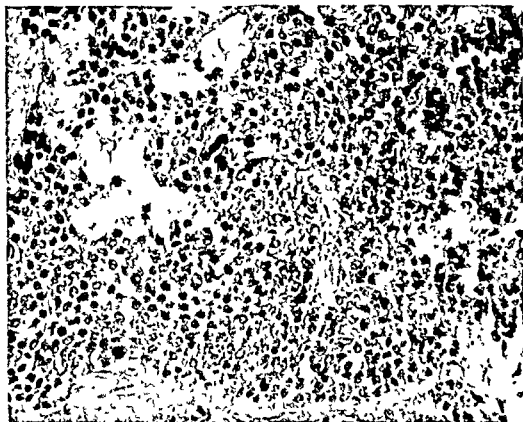
FIG. 3 —Another area of the testicular tumour under high power to show
the less well differentiated structure.   ×600 (approx).
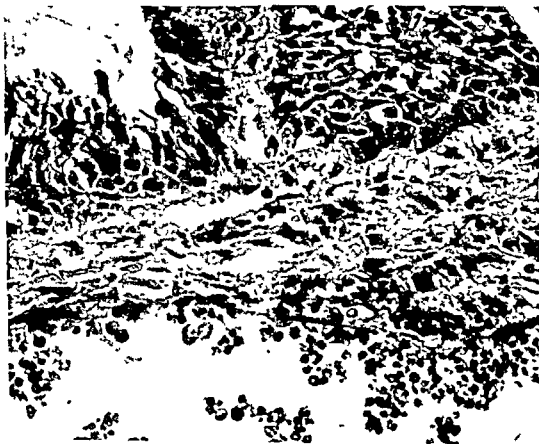
FIG. 4 —Enlarged prostate under high power to show metaplasia and
final shedding of epithelium.   ×600 (approx.)

size, causing urinary obstruction, and also in its histological appearance   This was from a dog with alopecia and pigmentation of the belly, atrophic testes, diminished spermatogenesis and inverted libido   Having again examined the material of de Jongh and Kok (1935), they pointed out that the structure was identical with that found in experimental stimulation with œstrin   They contrasted it with the more common type of prostatic enlargement in dogs, which is much less uniform, where there is glandular hyperplasia without metaplasia, the organ is not as large and there is no retention of urine, alopecia or pigmentation   In the present case, therefore, the dog shows all the features of general œstrogenic stimulation, with the possible exception of hypertrophy of the mammæ which arises in that state

If, therefore, one is to assume a connection between the prostatic enlargement and the testicular tumour in the present case, two possibilities arise   Either the tumour has arisen as a result of œstrogenic stimulation of the tissues of the testis, or the tumour is itself the source of œstrogenic substances

In the series collected by Kruckmann no feminisation was noted in patients bearing this tumour in a testis, but definite masculinisation appeared in those with one in an ovary   Kruckmann suggests that while the tumour may not be itself the origin of virilising hormones, it may arise from the action on the ovary of such substances elaborated elsewhere   The present case lends no support to such a hypothesis   However, many experiments have been performed by the injection of œstrogens into animals, including dogs, monkeys and man (e g de Jongh and Kok, 1935 , Zuckerman and Parkes, 1936)   The production of tubular adenoma in the course of such experiments does not seem to have been recorded

The question of the production of sex hormones by testicular tumours has been investigated, but those found were the gonadotropic and chorion stimulating, which are found in the urine , cases of tubular adenoma do not seem to have been studied (Ferguson, 1934 , Hamburger, Bang and Nielsen, 1936 , Gordon Taylor and Till, 1938)

There appears, therefore, to be no direct evidence of the production of œstrogens by this tumour, but there remains the fact that in dogs the characteristic features of œstrogenic stimulation—alopecia and pigmentation of the abdomen, sometimes with inversion of sexual behaviour—occur also with tubular adenoma of the testis (Innes)

Finally there must be considered the possibility that a tumour as large as the present instance, especially when occurring with mal development of the other testis, may so destroy the interstitial cells of the testis that the œstrogenic type of hypertrophy of the prostate may occur through suppression of production of androgens

Summary

A case is described of tubular adenoma of the testis in a dog, associated with the œstrogenic type of hypertrophy of the prostate   There is some evidence that this type of testicular tumour may produce androgens, but none that it may produce œstrogens, or that it is itself the product of œstrogenic stimulation   However, alopecia and pigmentation of the belly with inversion of sexual behaviour accompany both œstrogenic stimulation and tubular adenoma of the testis when these occur separately   Pigmentation and alopecia were present in this case   Relative overproduction of œstrogens might be caused by destruction by the tumour of the interstitial cells of the testis, especially when the other is mal developed

REFERENCES

FERGUSON, R. S. . . . . . 1934. *J. Urol.*, xxxi, 397.

GORDON-TAYLOR, G., AND TILL, 1938. *Brit. J. Urol.*, x, 1.
A. S.

HAMBURGER, C., BANG, F., AND 1936. *Acta path. et microbiol. Scand.*,
NIELSEN, J.   xiii, 75.

INNES, J. R. M. . . . . . 1942. *This Journal*, liv, 485.

DE JONGH, S. E., AND KOK, D. J. 1935. *Acta brev. neerl. Physiol.*, v, 177.

KRÜCKMANN, I. . . . . . 1936-37. *Arch. path. Anat.*, ccxcviii, 619.

MASSON, P. . . . . 1923. Tumeurs (pt. 2 of vol. xxvii of
                                               Sergent, Ribadeau-Dumas and
                                               Babonneix's "Traité de path-
                                               ologie médicale "), Paris.

PICK, L. . . . . 1905. *Arch. Gynaek.*, lxxvi, 191.

  ,, ... 1916. *Berl. klin. Wschr.*, liii, 1173.

ZUCKERMAN, S., AND GROOME, 1937. *This Journal*, xliv, 113.
J. R.

ZUCKERMAN, S., AND PARKES, A. S. 1936. *Lancet*, i, 242.

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TISSUE REACTION TO SERICITE AND SHALE DUSTS TREATED WITH HYDROCHLORIC ACID : AN EXPERIMENTAL INVESTIGATION ON THE LUNGS OF RATS

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(PLATES XLI AND XLII)

Since the publication of Jones's (1933) original communication on the presence of sericite in the silicotic lungs of coal-miners, there has been recurrent controversy over the question of the pathogenicity of this mineral. There can be no doubt about the large amounts of sericite in the lungs of workers from the S. Wales coal-fields (King and Nagelschmidt, 1945), and of its high concentration in the air-borne dust in the mines ; but although the balance of evidence appears to be in favour of quartz as the noxious agent in mine dust, it cannot yet be said with certainty that sericite is harmless or of low pathogenicity.

Like the evidence based on the study of human pneumoconiosis, animal experiments have also been equivocal. Several authors have reported minimal reaction of the tissues (lung and others) to the presence of the powdered mineral (Fallon and Banting, 1935 ; Lemon and Higgins, 1935 ; Miller and Sayers, 1936 ; Gardner, 1938 ; Simson and Strachan, 1940). Others have found it to produce pathogenic effects in the lungs (Drinker, Field and Drinker, 1931 ; Policard, 1934 ; Cummins, 1937 ; Selter and Weiland, 1937-38). Belt and King (1945) obtained the anomalous result that one set of sericite dusts (prepared from S. Wales shales) was almost without effect in animal lungs, whereas another sericite (from Ogofau, Carmarthenshire) produced a fibrous reaction almost comparable with that obtained with quartz.

The only plausible explanation of this last-named discrepancy lay in the fact that the fibrosis-producing sample of sericite had been subjected to both acid and alkaline treatment, for purification purposes, during its preparation. The other sericites, which produced the absolute minimum of tissue reaction, had suffered no other treatment than suspension in water during the course

of their separation by a settling process from the other minerals contained in the powdered shales from which they were prepared. One of us (King, 1945) noted a marked difference in the yield of dissolved silica in buffer solution and in plasma as between the Ogofau sericite and the S Wales sericites. The former was much more soluble than the latter. Treatment of the non pathogenic insoluble sericites with acids rendered them very soluble. A dust which had released only about 1 mg of SiO_2 into 100 ml of solution gave up 10 times as much silica, or more, after it had had preliminary treatment with hydrochloric acid. This increase in soluble silica, released from the sericite, may have been due to an opening up of the crystal lattice of the mineral particles exposing the silicon atoms and making them more available for solution in the surrounding liquid. Such a change might result in the dust's acquiring pathogenic properties which it did not previously possess. The present experiments were designed to test this hypothesis

Several specimens of S Wales sericite, from anthracite and steam coal mines, were injected into animals' lungs both without and with previous treatment with hydrochloric acid. In addition, the parent shales (mixtures of kaoli and mica (sericite) with a little quartz) were similarly investigated. Both shales and sericites gave minimal tissue responses in the untreated state and medium and marked responses after they had been treated with acid

MATERIALS

The sericites used in these experiments were the "secondary micas" or "illites" of the S Wales shales, the strata which overlie the coal seams. They form the bulk of most of these shales, and constitute a large proportion of the siliceous material in the dust breathed by workers in the coal field. Chemically, illite is a hydrated potassium aluminium silicate containing small percentages of iron, magnesium, calcium, sodium and titanium. The isolation by water sedimentation, and the full mineralogical and chemical descriptions are given by Hicks and Nagelschmidt (1943). In their report, and in that by Belt and King, they are referred to as the "fine fractions". We are indebted to Dr G Nagelschmidt for the samples used. They were from two anthracite mines and one steam coal mine

The parent shales, from which the sericites were prepared, were also used for animal experiments. The dusts were made by tumbling pieces of the rock in a rubber lined mill, as described by King

The size distribution of the shale dusts is given by Bedford and Warner (1945), all particles were under 5 μ and 80 per cent were under 1 μ. In the case of the sericite dust, all particles were under 1 μ

METHODS

Acid treatment of samples of each of the shale and sericite dusts consisted in suspending 1 g of the powder in N hydrochloric acid (100 ml) and shaking the mixture for 24 hours at room temperature or boiling it for 2 hours. The dusts were recovered from the acid by prolonged centrifuging and were well washed with water

Animal experiments were performed by the technique of Kettle and Hilton (1932). Full grown rats (200 g or more in weight) were lightly anæsthetised with ether, the trachea exposed by blunt dissection and 50 mg of the dust in 1 ml of saline (mixture sterilised by steaming) injected into the lungs. The operation is described in full by Belt and King. Twenty five animals were used in testing the acid treated dusts and 28 the non acid treated. Their survival time ranged from a few days to over a year. Any rats still alive were then killed

The lungs were removed, fixed and examined histologically as previously

described. Five slides were prepared from each block. One was stained with hæmatoxylin and eosin, one with van Gieson's stain and one by Gordon and Sweets's (1936) modification of Wilder's reticulin method; two were micro-incinerated, one being treated with hydrochloric acid to remove all but the siliceous residue. By comparing the incinerated slides with those which had been stained, it was possible to relate the position and distribution of the mineral dust in the section to the tissue changes observed.

RESULTS

Silica solubilities

These were determined by methods already described and are given elsewhere (King). The remarkable increase in the ease with which both sericites and shales release silicic acid into solution after they have been treated with mineral acid is seen in all the dusts but one. Sericite A3 more than doubles its solubility after acid treatment, but the final figure is still very small, and is only about a tenth that obtained with the other specimens. Moreover, this acid-treated material, though manifesting definite pathogenic properties, is still no more soluble than some of the other untreated dusts which produced the very minimum of tissue reaction. This curious discrepancy is yet another anomaly in the application of the solubility theory of silicosis as applied to the South Wales pneumokoniosis-producing dusts (cf. King).

Histology

Animals insufflated with sericite A3 (not treated with HCl). The lung tissue was very little disturbed by the presence of the foreign material. The dust lay in patches which seemed to correspond roughly to single lobules and their alveolar ducts. The only tissue reaction which was evident at the stage of 7-8 weeks was one of phagocytosis. Large numbers of macrophages had gathered up the dust particles and these packed cells occupied the alveolar spaces, lay in the alveolar walls and were crowded in the lymphatic spaces around the bronchi and blood vessels. A heavy deposit of phagocyted dust was present in the lymph nodes. Fixed-tissue reaction was not evident in any of the sites in which the dust was held up, nor was there any inflammatory-cell reaction (figs. 1 and 2).

Sericite A3 treated with HCl. In contrast to this picture, the animals in the group which received sericite treated with HCl showed a remarkable *reaction*, even as early as 5 weeks (figs. 3 and 4). The essential lesion was a nodule formed by fixed-tissue-cell proliferation about groups of dust phagocytes. The nodules were chiefly round or ovoid, but occasionally stellate, with tapering processes extending along the alveolar walls. The size of the nodules appeared to vary directly with their dust content and with the interval following insufflation. The smaller ones of 5-7 weeks' duration occupied a space roughly comparable to 1½-2 alveolar spaces of the rat's lung. The largest nodules occupied 3-4 alveolar spaces and were found in an animal which survived for 15 months. Confluence of nodules was marked in the peribronchial regions, but in the periphery of the lobules nodules were mainly discrete. In the nodules, groups of dust cells were incorporated into an organised mass of cells, the reaction being of the carnification type. In many nodules a faint concentric arrangement of the organising fibrils could be detected. Occasionally a moderate foreign-body reaction was evident. Fibrosis as measured by the amount of reticulin formation revealed by Wilder's reticulin stain was definite, but in moderate amount (cf. figs. 5 and 6). By the standards laid down by Belt and King, the reaction was classified as grade 3 in the older lesions, and grade 1 or 2 in the younger.

TISSUE REACTIONS TO SERICITE
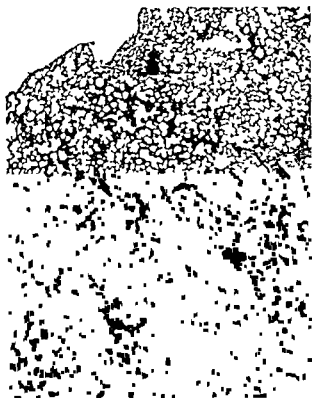
FIG 1—Sericite (illite) from anthracite mine A3   Dust untreated with HCl before insufflation   Duration of experiment 8 weeks   Dust is phagocyted and lies in alveolar walls and spaces   No inflammatory or fibrotic reaction   ×10
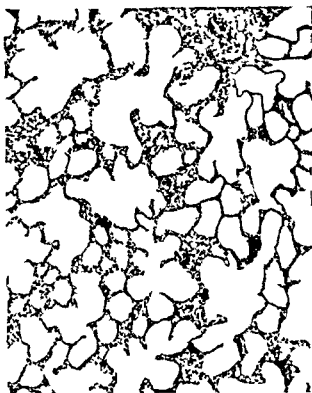
FIG 2—From the same slide as fig 1 Groups of dust laden phagocytes lie in the alveolar walls but no fixed tissue reaction is evident with dust not treated with HCl   ×80
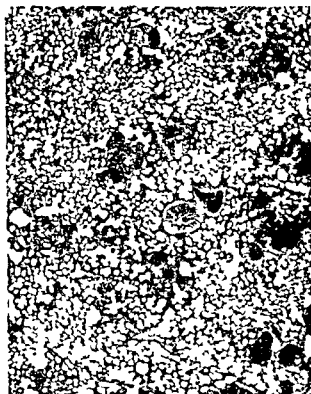
FIG 3—Sericite (illite) from anthracite mine A3   Dust treated with HCl before insufflation   Duration of experiment 5 weeks   Lesion consists of nodules of fibrous tissue with slight foreign body reaction   ×10

FIG 4—From the same slide as fig 3 Coarse nodules of dense fibrous tissue formed about accumulations of dust treated with HCl   Some confluence of nodules   ×80

TISSUE REACTIONS TO SPIRICITE
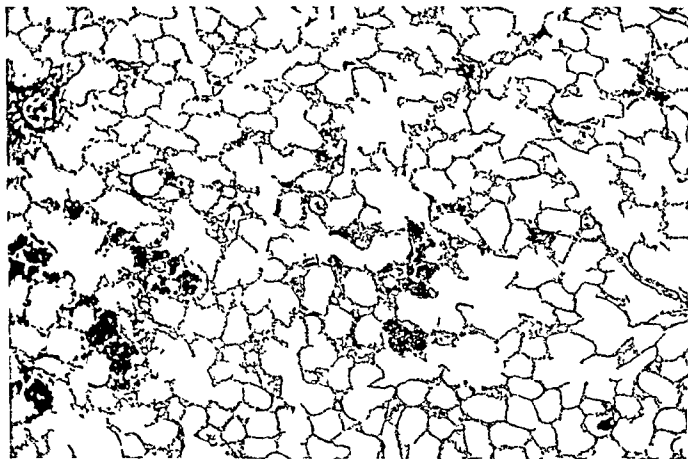
Fig 5 —From the same lung (after untreated dust) as figs 1 and 3   Wilder's reticulin
stain   ×80
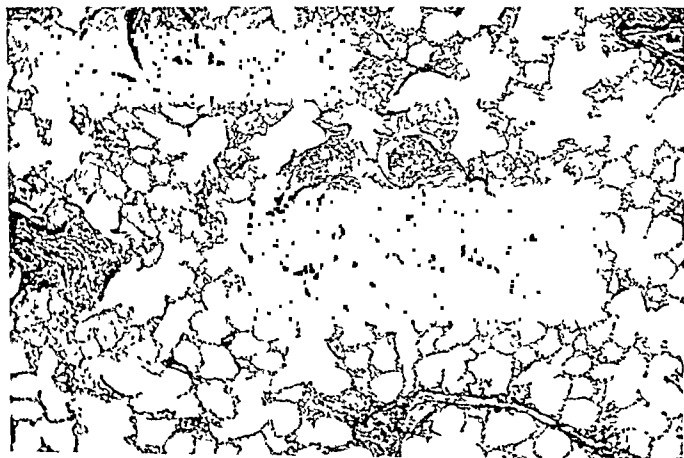
Fig 6 —From the same lung (after HCl treated dust) as figs 2 and 4   Wilder's reticulin
stain.   ×80

Sericite from mine S4 similarly appeared quite innocuous to rats until it was treated with HCl. It then produced organising nodules about groups of dust phagocytes, with fibrosis which was definite but not as intense as with the A3 dust.

Sericite from mine A1. The innocuous nature of this dust was confirmed, but the series with acid-treated dust was too small to permit any conclusions to be drawn

Shale (clod) from stratum C of mine A3 when treated with HCl produced a nodular fibrosis about the dust cells similar to the sericite. Untreated shale was phagocyted but caused no fixed tissue reaction

Shale from mine A1 stimulated no reaction in the lungs other than phagocytosis until it was treated with HCl : it then resulted in fine nodulation, with fibrosis rather less than that observed with the A3 shale.

SUMMARY

The innocuous nature of South Wales shales and sericites in the rat lung is confirmed. Preliminary treatment of shale and sericite dusts with hydrochloric acid resulted in their acquiring pathogenic properties, which led to the production of fibrous nodules in the lungs of rats.

REFERENCES

BEDFORD, T., AND WARNER, C. G.	1945.	Medical Research Council, Spec. Rep. Ser , no. 250, *London*, p. 88.
BELT, T. H , AND KING, E. J. .	1945.	*Ibid.*, no. 250, London, p. 29.
CUMMINS, S. L.	1937.	*Brit. J. Exp. Path* , xviii, 395.
DRINKER, C. K., FIELD, M. E , AND DRINKER, P.	1934.	*J. Indust. Hyg.*, xvi, 296.
FALLON, J. T., AND BANTING, F. G.	1935	*Canad. Med Assoc. J.*, xxxiii, 404.
GARDNER, L. U.	1938.	*Amer. Inst. Min. Met. Eng. Tech. Publ.*, no. 929.
GORDON, H , AND SWEETS, H. H , Jr.	1936.	*Amer. J. Path.*, xii, 545.
HICKS, D., AND NAGELSCHMIDT, G.	1943.	Medical Research Council, Spec. Rep. Ser , no. 244, *London*, p. 153.
JONES, W. R.	1933.	*J. Hyg.*, Camb., xxxiii, 307.
KETTLE, E. H., AND HILTON, R. .	1932.	*Lancet*, i, 1190.
KING, E. J.	1945.	Medical Research Council, Spec. Rep. Ser., no. 250, *London*, p. 69.
KING, E. J., AND NAGELSCHMIDT, G.	1945.	*Ibid.*, no. 250, London, p. 1.
LEMON, W. S., AND HIGGINS, G. M.	1935.	*Amer. Rev. Tuberc* , xxxii, 243.
MILLER, J. W., AND SAYERS, R. R.	1936.	*Publ. Hlth. Rep.*, li, 1677.
POLICARD, A.	1934.	*J. Indust. Hyg.*, xvi, 160.
SELTER, H., AND WEILAND, P. .	1937-38.	*Arch. Gewerbepath. Gewerbehyg* , viii, 71.
SIMSON, F. W., AND STRACHAN, A. S.	1940.	*Publ. S. Afr. Inst. Med. Res.*, no. xlv, p. 95.

616 . 145 . 13—007 . 64

A CASE OF ANEURYSM OF THE VEIN OF GALEN

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(Plates XLIII and XLIV)

Reading the reports by Russell and Nevin (1940) and Alpers and Forster (1945) on congenital arteriovenous aneurysm of the great vein of Galen, I have been struck by the remarkable similarity existing between these cases and one I encountered in 1939, which was considered at the time to be probably a congenital aneurysm of the great cerebral vein. Since these authors reported in all only 3 cases, and as they were unable to find other recorded examples, I venture to publish the following account of the case seen 8 years ago.

Case report

Clinical details. A. S., male aged 7 months, was admitted to the Royal Aberdeen Hospital for Sick Children on 9th June 1939, having been referred by the family doctor "on account of fits". The father and mother were alive and well, aged 24 and 25 years respectively. There was one other child, a girl aged 2 years, apparently healthy. In the present case, the pregnancy had been normal but the labour was rapid—said to have lasted only a quarter of an hour. At birth the baby was healthy and weighed $8\frac{1}{2}$ lb. It was never breast fed. In March 1939, at four months, it had slight fits with stiffening of the body and rolling of the eyes. These occurred over a period of only two or three days. In May 1939 the child developed a cold from which he recovered quickly. A recurrence on 6.6.39 was accompanied by a severe cough but no vomiting and very little general upset. On 9.6.39, about 4 p.m., the child's body became very stiff and the eyes glazed and staring. This lasted a few seconds and later recurred once or twice, but there were no twitchings or convulsive movements. The face became very pale.

On examination, the temperature was 101° F. and the pulse 140 per minute. The child was comatose and looked very ill. He showed marked hypertonia and lay in the position of opisthotonus, thoracic spinal rigidity being more marked than cervical. There was no response to painful stimuli, tendon jerks were much exaggerated, the eyes showed coarse nystagmus and there was intermittent strabismus. The pupils were very small and did not react to light. The head was of normal shape, but the fontanelles were bulging and showed marked pulsation. The ear drums were healthy.

The cardiovascular system was negative on examination. Breath sounds were vesicular and of a rather higher pitch at the left base, with rhonchi in both lungs. The abdomen was flabby and lax; liver and spleen were not enlarged.

Lumbar puncture showed the cerebro-spinal fluid to be under greatly increased pressure and brightly blood-stained.

The child died at 1.50 a.m. the following day.

Post-mortem summary. Subarachnoid hæmorrhage. Subacute otitis media. Patch of bronchopneumonia in posterior part of right lung. No signs of disease or anomaly in heart, kidneys, liver .spleen, adrenal glands or alimentary canal.

Description of the brain

The subarachnoid space contained fluid blood which extended over the cerebral hemispheres and over the base of the brain into the spinal subarachnoid space. The brain surface was intact. Blood welled out, on compression of the organ, from the region of the foramina related to the fourth ventricle. In the sagittal sulcus between the cerebral hemispheres was a large collection of fluid and clotted blood. This had manifestly come from a short rent, a few mm. long, in the wall of a roughly pear-shaped or sausage-shaped sac which lay amongst blood clot on the upper surface of the tentorium cerebelli, cushioned between the cerebral hemisphere and the posterior part of the falx cerebri. After removal of the surrounding blood and blood clot, the sac was found to be free at its bulbous postero-superior extremity, but on its narrow antero-inferior extremity it was firmly attached by a neck or pedicle, under the splenium of the corpus callosum, apparently to the falx cerebri at its junction with the tentorium cerebelli, *i.e.* in the vicinity of the confluence of the great vein of Galen with the inferior sagittal sinus and straight sinus. The whole of the region was obscured by fluid and clotted blood. A tenuous filament of tissue resembling a small vessel passed from this pedicle into the third ventricle, where it was lost in the choroid plexus.

The sac measured 3·5 cm. in length and 1·5 cm. in its broadest diameter. Fig. 1 is an actual-size outline tracing made after bisection of the specimen.

The sac was filled with fluid blood and soft blood clot. Its wall was fairly thick (as in fig. 1), measuring just over 1 mm. in thickness. There was no lamination of the blood clot. The external surface of the sac, apart from recently adherent blood, was quite smooth, pale pink-grey and semi-translucent, so that the contents were recognisable from the outside as probably blood or blood clot. On palpation, the mass was turgid, and gentle compression led to the extrusion of dark blood from the rent in the wall.

Fig. 1. — Aneurysm of vein of Galen. Cellophane tracing of longitudinally bisected specimen.

The ventricular system of the brain was filled throughout with recent blood. There was a moderate degree of hydrocephalus involving the lateral and third ventricles, the latter being conspicuously distended and forming an almost spherical sac. The aqueduct of Sylvius and the fourth ventricle, though filled with blood, were not appreciably distended.

No associated congenital malformations were seen elsewhere in the brain or in the intracranial vessels.

The foregoing description is taken from my original report and apart from some condensation has not been materially altered in retrospect. Paraffin blocks of the aneurysm and of several parts of the brain were preserved but were not examined till recently.

Histological examination

Paraffin sections from the wall of the aneurysm were stained with hæmatoxylin and eosin, hæmatoxylin and van Gieson, Masson's hæmatoxylin-fuchsin ponçeau-light green method, Weigert's elastic stain, and mucicarmine.

The blocks from the brain came from the region surrounding the posterior horn of the lateral ventricle, the frontal and occipital lobes, the structures forming the walls of the fourth ventricle, the lower temporal region showing

the hippocampal gyrus, the vessels in the transverse fissure and part of the choroid plexuses of the fourth and lateral ventricles. All these sections were stained by H. and E. only.

Microscopically (fig. 2) the sac has a definite wall resembling that of a vein but much thickened. Beneath a thin outer covering of collagenous tissue (with which extravasated blood cells are intermingled) there is a narrow band of circular smooth muscle of fairly healthy aspect. Inside this muscularis the rest of the wall, constituting more than three-quarters of its width, consists of a nondescript myxoid tissue of finely fibrillated character. Whether this is hyalinised thickened intima or a degenerated muscularis it is impossible to say. Here and there frankly mucinous change is seen in sections stained with mucicarmine. In this myxoid or hyaline layer the nuclei are scanty, thin and elongated, with a fairly dense chromatin arrangement. The thin wavy cytoplasmic prolongations which constitute most of the structure of this zone stain yellow with van Gieson's stain and red with Masson's trichrome stain. The contents of the sac consist simply of recent thrombus, with some layering into red corpuscles, leucocytes and fibrin, but there is as yet no sign of reaction nor any attempt at organisation. There is no demonstrable elastica.

The sections from the brain reveal marked dilatation of the capillaries of the choroid plexus of the lateral ventricle (fig. 3), contrasting markedly with the choroid plexus of the fourth ventricle, where this feature is absent (fig. 4). There is gross dilatation also of a small group of capillaries (fig. 5) just under the ependyma of the lateral ventricle towards the posterior part of the lenticular nucleus. Dilatation of small vessels appears to be restricted to the regions indicated, for it is not seen in the blocks from the other regions of the brain.

Comment

Most attention at the time of autopsy had been directed to the aneurysmal sac itself, since it appeared to be a local and restricted condition, and since at that time the case had for me no precedent. Yet in retrospect there seems little doubt from the microscopical examination of the material conserved, incomplete as it was, that here too there must have been a true arteriovenous communication though of a much less gross nature than in case 1 of Russell and Nevin or in the case described by Alpers and Forster. The present case appears to resemble most closely Russell and Nevin's second case.

Having regard to the fact that the description of the aneurysm in the present case was written before the reports on the other 3 cases were available, it is interesting to observe its striking similarity to the others. Russell and Nevin's case 2—that of a child of 17 months—is described as follows (p. 380): "The vein of Galen was replaced by a saccular aneurysm, 3·5 cm. in diameter, which occupied a concavity in the depressed corpora quadrigemina. The aneurysm was attached to the ventral point of junction of the falx with the tentorium, while the pineal body . . . lay on its ventral surface". Alpers and Forster (p. 182) described the aneurysm in their case—that of a youth of 18— as lying "between the cerebral hemispheres, occupying the pineal recess and resting on the tectum mesencephali. It measured 4 by 2·5 by 2·5 cm. It was dome-shaped, its broadest portion lying superiorly, with a narrow waist at the point of union with the vessels of origin. The walls were firm and dense but measured only 2·5 to 3 mm. in thickness. Dissection revealed that the body of the aneurysm arose from the junction of the great cerebral vein (Galen) with the straight sinus, but it was not possible to determine which of the vessels contributed most to its formation ".

PLATE XLIII

ANEURYSM OF VEIN OF GALEN
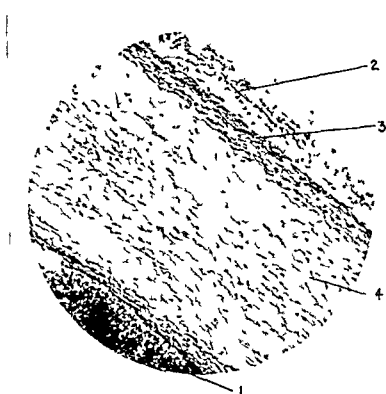
FIG 2 —Wall of aneurysmal sac   Masson s trichrome stain
1 Blood in lumen   2 Adventitia with intermingled red blood cells   3 Muscularis
4 Myxoid layer   ×70

FIG 3 —Choroid plexus of lateral ventricle with greatly dilated vessels   ×60

ANEURYSM OF VEIN OF GALEN

Fig 4—Choroid plexus of fourth ventricle for comparison with fig 3   ×60

Fig 5—Greatly dilated capillary vascular spaces under the ependyma of the lateral
ventricle   ×40

Summary

A case of aneurysm of the vein of Galen is described and is believed to be the fourth such case on record. The subject was a child of 7 months and death was due to rupture of the aneurysm. Hydrocephalus was present as in the other reported cases.

REFERENCES

ALPERS, B. J., AND FORSTER, 1945. *Arch. Neurol. Psychiat.*, liv, 181.
  F. M

RUSSELL, DOROTHY S, AND 1940   This *Journal*, li, 375.
  NEVIN, S.

616—006 . 449—031 . 73 : 611 . 829

A CASE OF CHLOROMA INFILTRATING THE SPINAL MENINGES

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Case report

R C , æt. 19, a miner, was admitted to University College Hospital under the care of Dr Walshe on the evening of 5th October 1945 with retention of urine and a flaccid paralysis of both legs. He had been working underground until the beginning of June 1945, when he returned home complaining of malaise and vague abdominal pain. He stayed in bed for a few weeks and by the end of July felt fit and was ready to return to work, but in the second week of August he had several short attacks of sharp shooting pains down his right leg and in his foot. He remained at home and in a few days the pain disappeared. The same pain recurred a fortnight later, and he was sent to bed and an injection given into his buttock Again the pain subsided and he started to get up, but a few evenings later, three weeks before his admission to U C.H , severe pain returned in the back of the right thigh and extended down the front of the leg and into the foot. He was kept awake by this pain, and his leg felt cold and heavy. Next morning he found he could not use the leg Five or six days later he suffered similar but less severe pain in the left leg, and within twenty-four hours had lost all movement of that limb also, and he began to have difficulty in passing his urine. When he did manage to start the flow, he noticed that he had no sensation of its passage. He found also that he suffered little discomfort, even when his bladder was full.

There was little change in his condition until 2nd October, three days before his admission to hospital, when he began to suffer severe aches and shooting pains in the small of the back and across the upper part of the abdomen and the lower ribs anteriorly The pain became sharp and exquisite if he coughed or lifted his head Next day the skin of the outer part of the eyelids of the left eye became purplish as if bruised, although he had had no blow. He remembered that a week before some bruises had appeared spontaneously on his legs.

On admission, his temperature was 101, pulse rate 140 and respiratory rate 22. He was seen to be very pale and poorly built. There was blue-black discolouration, without swelling, of the skin on the outer part of both eyelids of the left eye, extending laterally to the margins of the orbit. There were some faded areas of brown discolouration on the dorsum of both feet.

There was flaccid paralysis of both legs, with absent reflexes, and *complete sensory loss* extending upwards to a sharply defined line of demarcation at the *level of the umbilicus.* No abnormality was detected in the arms or in the cranial nerves.

Small brownish flecks could be seen in both retinæ, lying in the mid-line just below the discs. These were thought to be hæmorrhages, but Mr Neame later pronounced them to be a congenital abnormality.

The bladder was grossly distended, yet the patient suffered no discomfort. A catheter was passed and left *in situ*, the bladder being drained slowly. When the abdomen was examined later, neither spleen nor liver was palpable. No enlarged lymphatic glands were found, although the tonsils were slightly hypertrophied.

The following morning, 6th October, the line of sensory loss had risen to midway between the xiphisternum and the umbilicus, and the ecchymosis around the left eye had spread slightly.

A blood count revealed the following picture :—

Hæmoglobin	38 per cent.	
Red blood corpuscles . . .	2,000,000 per c.mm.	
White blood corpuscles	47,000 ,,	,,
Myeloblasts	30,500 ,,	..
Myelocytes	3700 ,,	,.
Neutrophils	11,800 ,,	,,
Lymphocytes	1000 ,,	,,

A diagnosis of acute myeloid leukæmia was made and it was thought that the ascending myelitis was due either to hæmorrhage into the cord or to deposits of leukæmic tissue in or around the cord.

On 7th October several small fresh petechiæ were found on the abdomen and on the dorsum of each foot. The line of sensory loss had risen to the level of the nipples and the arm jerks were found to be less brisk on the right side than on the left. His temperature rose at mid-day to 104° F. and on this and subsequent days until his death there were severe rigors. His sleep was disturbed by hiccough. On 9th October he was very drowsy until the evening, when he seemed remarkably cheerful and alert. Lumbar puncture was performed that day. Attempted at the usual level it was unsuccessful, but when tried at the L. 2-3 level a bright yellow fluid was obtained under low pressure, which clotted almost as soon as it was drawn. No leukæmic cells could be seen in sections of this clot made after fixation.

During the night of 9th-10th October his temperature rose to 105·8 and he shivered and sweated. Next day his breathing was very shallow and he was semi-comatose. The pulse was almost imperceptible and remained so until his death on the morning of 11th October.

At post-mortem it was seen that there was little subcutaneous fat and that the muscles were wasted. Externally ecchymoses were present over the left eye and left iliac crest. Internally very little abnormal was found until the vertebral canal was opened up. There was no sign of leukæmic infiltration in the lungs, which were congested and œdematous, or in the heart or pericardium. The liver was congested and the spleen distended, but neither showed any leukæmic infiltration ; no enlarged lymph glands were found, but the tonsils and lymph nodes at the base of the tongue were prominent. The pituitary, thyroid and adrenals seemed normal. In the kidneys small round white areas

of leukæmic infiltration were seen on stripping the capsule; otherwise these organs were normal. The upper two thirds of the marrow cavity of the femur contained greenish black diffluent tissue, the nature of which was obscured by post-mortem change.

When the vertebral canal was opened, masses of green chloromatous tissue were seen growing from the laminæ of the lower dorsal vertebral bodies and occupying the extradural space; these were compressing the cord between the levels of the 3rd and 8th dorsal vertebræ. The bodies and laminæ were not softened, but were partially infiltrated by the growth, which also extended into the transverse and spinous processes, and in places had spread outside the vertebræ into the ligaments on the lateral surfaces of both bodies and discs. In some places the chloromatous tissue had spread along the ribs and could be seen through the parietal pleura, to which it imparted a greenish sheen. The manner in which the tumour swelled from the spinal canal when the laminæ were removed indicated the considerable pressure to which the under lying cord must have been subjected.

Histologically the tumour was composed of closely packed myeloid cells. the majority of which were early myelocytes.

Discussion

A description of a chloroma was given by Allen Burns of Glasgow in 1824. His brother attempted unsuccessfully to remove a "fungus hæmatodes" growing in the right orbit. At post mortem he found extension of the yellowish green tumour tissue through the bones of the basicranium into the paranasal sinuses, infiltration of the dura mater, and greenish tumour masses eroding the skull. Since that time much has been written on the subject of these interesting tumours and there has been much controversy as to their origin and about the nature of the green pigment which is their characteristic feature (Sternberg, 1904-05 ; Brannan, 1926).

Although much attention has been given to the neurological manifestations of leukæmic diseases, interest has been mainly confined to the cerebral lesions, and lesions of the spinal cord have not received due consideration. Critchley and Greenfield (1930 31) have described several types of lesion which may occur. Sometimes damage is caused by leukæmic cells which infiltrate the cord, either diffusely or locally in patches. Occasionally perivascular infiltration reduces the blood supply and causes local myelomalacia or softening of the cord. Rarely there is hæmorrhage into the cord or into the subarachnoid space. Chloromatous infiltration of the meninges is most commonly found, and Critchley and Greenfield describe 12 such cases, 5 of which were leukæmic in nature. Such infiltration either directly compresses the cord or interferes with its blood supply. Some workers have speculated on forms of subacute combined degeneration of the cord which they state may occur in certain leukæmias and are caused by obscure toxins.

In the case here described there was infiltration of the spinal meninges, and the chloromatous masses were under very great pressure inside the vertebral canal and were compressing the cord. The most interesting feature of the case is that a disease so closely related to the blood dyscrasias should manifest itself by neurological symptoms only, almost till death.

REFERENCES

BURNS, A. . . . . 1824. Observations on the surgical ana-
tomy of the head and neck,
2nd ed., *Glasgow*, pp. 384-392.

BRANNAN, D. . . . . . . 1926. *Bull. Johns Hopkins Hosp.*, xxxviii,
189.

CRITCHLEY, M., AND GREENFIELD, 1930-31. *Brain*, liii, 11.
J. G.

STERNBERG, C. . . . . . . 1904-05. *Beitr. path. Anat.*, xxxvii, 437.

576.8.093.4

A METHOD FOR COMBINED POSITIVE AND NEGATIVE STAINING OF BACTERIA

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(PLATE XLV)

A simple and reliable method of combining positive and negative staining of bacterial films would have many applications. Unfortunately, as Fleming (1941) has admitted, the best of the known negative stains, nigrosin, so rapidly decolourises the ordinary basic dyes that it is difficult to combine its effect with positive staining of the bacterial bodies. Seeking a more compatible mating of stains, I have found that positive staining with methylene blue or other such dye may readily be superimposed on bacterial films prepared by the congo-red method suggested by Benians (1916) for " relief staining " of bacteria and spirochætes and subsequently used by Hort (1915-17) for the exhibition of problematic forms in bacterial cultures.

Method

1. Free the slide from grease by heating it in the Bunsen flame, after which it may be quickly cooled by laying it on a smooth glazed tile.
2. Place on the slide a suitable drop of an almost saturated aqueous solution of congo red containing about 10 per cent. of serum and mix in a particle of the agar-grown culture to be examined.
3. Spread the mixture in a film of varied thickness, dry with gentle warmth and then fix thoroughly in the flame.
4. Flood the film when cooled with a 0·5 per cent. aqueous solution of HCl; drain, blot gently and drive off excess of acid with gentle warmth.
5. Stain the film for 15-20 seconds with a 1 per cent. aqueous solution of methylene blue, which may be acidulated with acetic acid—1 small drop of " glacial " acetic to 20 c.c. of stain.
6. Drain off the stain—do not wash—and blot the film gently but thoroughly; dry and examine, mounting first if there is any question of preserving the preparation.

To these instructions it need hardly be added that the stains and serum employed should be clean, bacteriologically and otherwise; that where *blood* or other serous material is examined no other addition of serum to the congo red is required; and that in the examination of broth cultures the *organisms*

COMBINED POSITIVE AND NEGATIVE STAINING OF BACTERIA
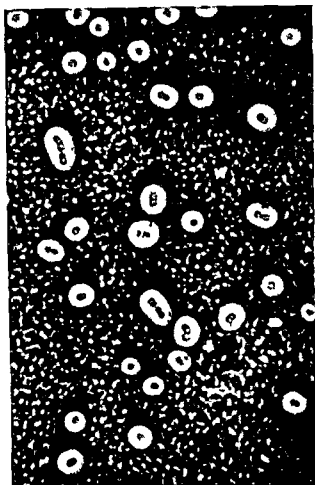
FIG 1 —Pneumococci in peritoneal fluid of
mouse   ×1250

FIG 2 —Capsulate and non capsulate
bacilli from a mucoid culture of B
anthracis   ×1250
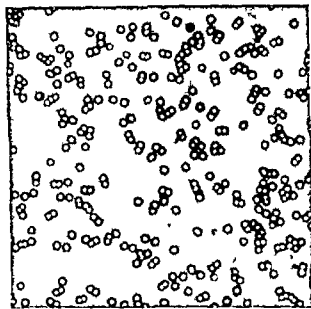
FIG 3 —Normal staphylococci from a
7 hours' agar plate culture   One dead
coccus appears as a dark point   ×1250
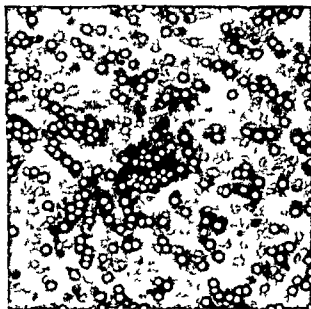
FIG 4 —Staphylococci from a 7 hours' plate
culture which at the end of the 3rd hour
was treated with 5 units of penicillin
Living cocci, stained blue, appear light,
dead cocci, stained brown, appear dark,
while the background of the film is
stippled with the poorly staining debris
of disintegrated organisms   ×1250

must first be separated from peptone, salts and so on, the water-solubility of which would threaten the integrity of the film. The process takes very much the same amount of time and labour as does Gram staining. The film, at first red, then rendered blue by the acid, assumes a deep purple tint through inter-action of the two dyes, the colour later maturing to red-purple on intervention of alkali from the glass.

Results

Viewed at the completion of this sequence the blue-stained bacterial bodies are seen against a field of rich orange or gold. The most decorative effects are obtained in the display of bacterial capsules (figs. 1 and 2), which appear as peribacterial haloes, usually unstained but sometimes violet-tinted and presenting signs of internal structure. Careful comparisons have shown that the appearances of capsulation presented may be confidently accepted as evidence of the fact. In a similar manner, provided their substance be not too generally dispersed, the negative stain exposes those accumulations of intercellular secretion which are associated with the formation of pellicles in broth cultures and of coherent, adherent and often corrugated growth on agar. In dense formation, unrarefied by uptake of water, this interstitial material often takes a violet stain. Spores, shed and unshed, present a characteristic appearance of refractile bodies sharply outlined (see White, 1946, figs. 3 and 4).

It was Henrici (1925) who first pointed out that dead bacteria, in contrast to living, are penetrated and stained by congo-red. The dual stain described is a ready and useful method of putting the contrast in evidence : organisms alive at the preparation of the film are blue ; those already dead stain brown or purple. By this means a reasonable presumption may be quickly reached as to the viability of an aged culture or as to the sterility of a vaccine. To take a topical application : the method, while admirably exhibiting the initial morphological changes wrought by penicillin in staphylococci (figs. 3 and 4), registers, as films are made from the stricken culture at intervals, the onset and spread of cell death to the point at which the few " persisters " lie scattered as blue points, intensely ringed by purple-staining secretion in a brown back-ground of bacterial debris.

The method therefore is broadly catholic in its service, supplying from a single preparation a wide range of information. I regard its reagents as a useful addition to the general purposes stains of the laboratory bench.

My thanks are due to Mr F. Welch of this Institute for the microphotographs reproduced to illustrate this note.

REFERENCES

BENIANS, T. H. C. . . . . 1916. *Brit. Med. J.,* ii, 722.
FLEMING, A. . . . . 1941. This *Journal,* liii, 293.
HENRICI, A. T. . . . . . 1925. *J. Inf. Dis.,* xxxvii, 75.
HORT, E. C. . . . . 1915-17. *Proc. Roy. Soc.,* B, lxxxix, 468.
WHITE, P. B. . . . . 1946. *Brit. J. Exp. Path.,* xxvii, 356.

578.65:612.112

AN IMPROVED METHOD OF STAINING LEUCOCYTE
GRANULES WITH SUDAN BLACK B

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(PLATE XLVI)

A method for the specific staining of the granules of the "granular" leucocytes and of the monocytes in blood films with Sudan black B was described by Sheehan (1939) and this stain has since been used by McManus (1945) and by Discombe (1946). The original method proved to be occasionally unreliable with war-time reagents, but the present modification gives very consistent and rather better results. It can be used equally well with new or old films of blood or bone marrow. The appearances are the same as were described in the previous paper. The polymorph leucocytes (figs. 1-3) are packed with fine granules, most of which appear to have slid off the surface of the nucleus when the cell flattened out on the slide, as if they had been lying loose in a somewhat fluid cytoplasm. The eosinophil granules (fig. 5) have a distribution in the cell similar to that seen in polymorphs. They appear as black circles owing to the possession of a rind which stains very easily and of a centre which is always unstained, presumably because of biochemical differences. The monocytes (figs. 3 and 4) usually have a moderate number of small granules which are distributed very evenly. The single layer of cytoplasm which is visible over the upper surface of the nucleus contains about half the number of granules that can be seen through the entire thickness of the cytoplasm at the side of the nucleus. This appearance might be produced if the granules were set fairly uniformly in a rather tough layer of cytoplasm near the surface of the monocytes. The azur granules of lymphocytes are unstained (figs. 1 and 2). Marrow cells and abnormal blood cells will be described in a subsequent paper, but it may be noted here that in certain blood diseases a number of the polymorphs have granules which are not sudanophil.

The improved technique is as follows :—

(1) The dry film is fixed with formaldehyde vapour by placing it for 5-10 minutes in a closed jar containing some 40 per cent. formalin in its lower part. This gives very satisfactory fixation of erythrocytes as well as leucocytes.

(2) The Sudan staining solution should have a neutral or slightly alkaline reaction ; if it is slightly acid it stains only the granules of eosinophil leucocytes. It is improved by the addition of phenol, which appears to have a mordanting action on the granules.

Stock buffer solution. Sixteen g. of crystalline phenol are dissolved in 30 c.c. of absolute alcohol. This is added to 100 c.c. of water in which 0·3 g. of $Na_2HPO_4.12 H_2O$ has been dissolved.

Stock Sudan solution. 0·3 g. of Sudan black B is dissolved in 100 c.c. of absolute alcohol. The dye must be completely dissolved. This can be ensured either by leaving the solution at room temperature for a day or two, with frequent shaking, or by grinding in a mortar and heating the resulting suspension.

For use, 40 c.c. of the buffer solution is well mixed with 60 c.c. of the Sudan solution and filtered by suction. This buffered stain is ready for use at once ; it can be used for several weeks, but becomes gradually slower in action with the lapse of time.

STAINING OF LEUCOCYTE GRANULES
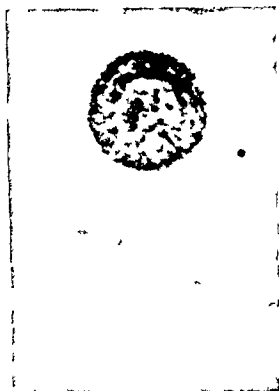
Fig 1.—Neutrophil polymorph and
large lymphocyte   ×1600

Fig 2.—Neutrophil polymorphs and
small lymphocyte   ×1600

Fig 3.—Neutrophil polymorph and
monocyte   ×1600

Fig 4.—Monocyte   ×1600

Fig 5.—Eosinophil   ×1600

The fixed film is immersed in the buffered stain in a covered jar for 10-60 minutes. The longer times are required if the original stock Sudan solution was not completely dissolved or if the final stain is old or very cold.

3. The slide is now well washed in absolute alcohol or 70 per cent. alcohol for a few minutes. The granules are only slowly decolourised by very many hours' treatment with either absolute alcohol or xylol, though more quickly with acid alcohol.

4. After washing in water, the slide is counterstained as desired. The most reliable stain is a 1 : 10 dilution of Gurr's Improved Giemsa R.66 in neutral water, allowed to act for 30 minutes. The blue tint of plasma and erythrocytes is then removed by differentiation for $\frac{1}{2}$-1 minute in a 0·2 per cent. aqueous solution of KH_2PO_4. The film can be examined in immersion oil or can be mounted in balsam.

REFERENCES

DISCOMBE, G. . . . . . . This *Journal*, 1946, lviii, 572.
McMANUS, J. F. A. . . . . *Nature*, 1945, clvi, 173.
SHEEHAN, H. L. . . . . . This *Journal*, 1939, xlix, 580.

A METHOD FOR STAINING GROSSLY FATTY TISSUES WITH SCHARLACH R

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In the course of an investigation carried out recently in this laboratory, it was necessary to prepare frozen sections stained with Scharlach R. of grossly fatty kidney and liver. Presumably because the amount of fat in these tissues was so great, the frozen sections prepared from them constantly adhered not only to one another but also to almost everything with which they came in contact, so that it was practically impossible to produce whole sections free from folds. These difficulties were overcome in the following way. Blocks of tissue, after fixation in formalin, were embedded in gelatin by Aschoff's method and frozen sections cut. The sections were taken into 50 per cent. alcohol and then transferred to a very dilute solution of gelatin, from which they were picked up on slides. The slides were exposed to formaldehyde vapour to fix the gelatin mountant, and the mounted sections stained. By this method the sections became easy to handle and were flat, and whole sections were readily obtained. The method is especially useful when many sections are needed either for research work or for classes of students.

Details of embedding and section-cutting

1. After formalin fixation, wash the tissue in running water for 24 hours.
2. Soak in 12·5 per cent. gelatin solution in the incubator at 37° C. for 24 hours.
3. Transfer to 25 per cent. gelatin solution at 37° C. for 24 hours.
4. Embed in 25 per cent. gelatin solution and place in refrigerator to set.
5. Place the block in 5 per cent. formalin solution for 24 hours.
6. Trim the gelatin block to within 1 mm. or so of the tissue and cut sections 10·15 μ thick on the freezing microtome.

7. Transfer sections from knife with a soft brush or the finger moistened with 50 per cent. alcohol to a dish containing 50 per cent. alcohol.

8. Transfer sections to 0·5 per cent. gelatin solution for a few seconds.

9. Pick up sections from the gelatin solution on clean glass slides. A finely drawn-out piece of glass rod is useful for steadying the sections during this manipulation. Drain off excess solution, taking care that sections do not dry.

10. Stand slides in a staining trough over formalin at 37° C. for at least 1 hour. The slides must not rest in the formalin. Pieces of glass rod placed across each end of the bottom of the trough raise the slides out of the liquid.

11. Add distilled water to the formalin already in the trough so as to submerge the sections and leave until staining is to be done.

Details of staining

1. Rinse sections in distilled water.
2. Transfer to 70 per cent. alcohol.
3. Stain in Scharlach R. for 3-5 minutes.
4. Rinse thoroughly in 70 per cent. alcohol.
5. Rinse thoroughly in distilled water.
6. Stain in Ehrlich's hæmatoxylin for 10 minutes.
7. Blue in running water.
8. Differentiate in acid-alcohol.
9. Blue again in running water.
10. Mount in a watery medium such as glycerine jelly or Farrant's medium.

Notes

1. All the gelatin solutions are made with distilled water and contain a little thymol to preserve them.

2. The Scharlach R. is a saturated solution of the dye in equal parts of 70 per cent. ethyl alcohol and acetone.

3. A very satisfactory modification of Farrant's mounting medium has been devised by G. W. Moore of the Central Histological Laboratory, London County Council. It contains :—

Picked gum arabic	100 g.
Pure cane sugar	50 ,,
Distilled water	300 c.c.
Thymol	0·2 g.
Glycerol	25 c.c.

Dissolve the gum arabic and cane sugar in the distilled water in the steamer. Cool, clear with the white of two eggs and filter through muslin. Add the thymol and finally the glycerol.

Comments

The use of 50 per cent. alcohol in removing the sections from the knife eliminates all risk of their sticking to the brush or finger.

When transferred to the 0·5 per cent. gelatin solution after immersion in 50 per cent. alcohol, the sections float to the top and flatten out so that they are readily picked up on the slides.

It is important that during the early stage of fixing the sections to the slides the latter should not touch the formalin. Should they do so the liquid will creep up the slides and tend to detach the sections.

The sections are purposely overstained with hæmatoxylin because the longer period of differentiation needed is then sufficient almost completely to decolourise the gelatin with which the sections are impregnated.

AN UNUSUAL CONGENITAL CARDIAC DEFECT: FAILURE OF
APPEARANCE OF THE ENDOCARDIAL CUSHIONS, THE AORTIC
SEPTUM AND THE DISTAL PART OF THE LEFT SIXTH
BRANCHIAL ARCH

R. E. REWELL [1]

The Zoological Society of London

The specimen here described was sent to me by Dr L. M. Traube of the
Beckenham and Penge Joint Maternity Hospital. It came from a male infant,
delivered normally after a normal pregnancy, who became cyanosed soon
after birth and survived only three days. No abnormal physical signs were
discovered in the chest. Dr Traube performed a necropsy, and finding a condition
with which she was not familiar sent the thoracic contents to me for further
study. No abnormalities were found outside the thorax.

Description of the specimen

On external examination the heart appeared to be of normal size and shape,
but there was only one vessel in place of the pulmonary and aortic trunks.
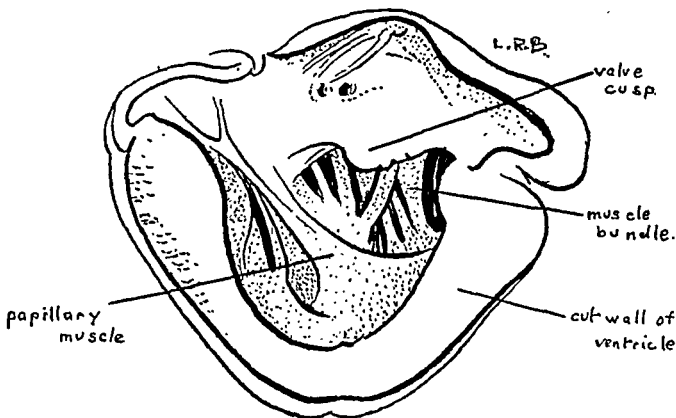
FIG.—Congenital cardiac malformation. Sketch of the specimen opened from the right
side to show the atrio-ventricular valve with its chordæ and papillary muscle and
the muscle bundles crossing the lumen of the single ventricle. × 2 (approx.).

This occupied the usual position of the pulmonary artery and gave branches to
the lungs, but became continuous with the aortic arch and gave off the innominate
and left common carotid and subclavian arteries in the manner usual to this
latter vessel.

Internally the right atrium and the openings into it of the superior and
inferior venæ cavæ and the coronary sinus appeared to be normal. On first
examination the interatrial septum also appeared normal from this side. The
atrioventricular orifice was guarded by a tricuspid valve which led into a single
ventricle with no trace of a longitudinal septum. Papillary muscles arose

from the anterior and right lateral walls of the ventricle. The chordæ tendineæ of the third cusp of the atrioventricular valve joined the posterior wall of the ventricle individually. The cavity of the ventricle was traversed by several columns of muscle resembling papillary muscles. Some ran upwards and backwards from the anterior wall, others upwards, forwards and to the left from the posterior wall.

The persistent truncus arteriosus described above led out of the ventricle and no trace of a dividing septum could be found within it. Its opening was guarded, however, by a tricuspid valve of the " watch pocket " type.

No remnant of the ductus arteriosus could be found, *i.e.* the distal part of the left sixth branchial arch appeared to be absent.

On opening the left atrium from behind, the portion into which the four pulmonary arteries opened appeared to be marked off from that nearer to the ventricle by a slight constriction, *i.e.* absorption of the common pulmonary vein did not appear to have been completed. The auricular appendage was normal. However, no opening was present between the left atrium and the common ventricle, the interatrial septum coming over close to the lateral wall of the atrium. There thus appeared to be no outlet from the atrium, which would have made it impossible for blood returning from the lungs to get back into the circulation. In the fœtus this might have been conceivable, but the child had survived for three days, so respiration must have been carried on by some means. Further search revealed a very narrow channel through the interatrial septum, opening into the right atrium low down in its wall. It is possible that in life this orifice was much wider than was apparent at autopsy.

Discussion

The anomalous interatrial septum. In the development of the normal heart during the fifth week of intra-uterine life, the sinus venosus opens into the right side of the common atrium, and a ridge, the septum spurium, runs down from the roof to become continuous with the venous valves guarding this orifice. Next the septum primum appears as another ridge in the roof just to the left of the septum spurium. In the sixth week, the atrioventricular canal becomes divided longitudinally by the fusion of the anterior and posterior endocardial cushions which thus form the septum intermedium. The common atrium becomes divided into two by the septum primum extending down to meet the septum intermedium.

In the present case it would appear that the endocardial cushions had never arisen. As a result, no septum intermedium was formed, and so the septum primum passed over to the left and attached itself to that margin of the atrio-ventricular canal.

An alternative explanation of the absence of the left atrioventricular canal is that this orifice had been closed as a result of inflammation. However, the interatrial septum comes over very close to the side wall of the left atrium and no pit or other obvious sign marks where the opening of the canal may have been. Moreover, there is no trace of any of the cusps of a mitral valve nor any chordæ tendineæ for them, nor do any of the muscle bundles crossing the ventricle appear to be in the correct position for the appropriate papillary muscles.

The absence of the interventricular septum. This septum is formed normally from three parts. The muscular portion arises from the septum inferius, which starts from the floor of the common ventricle and runs upwards to divide it longitudinally. Part of the membranous portion of the interventricular septum is formed from the proximal bulbar septum which divides the lowest part of the bulbus arteriosus longitudinally and so lies just anterior to the membranous part of the interventricular septum, so close that part of the latter is formed by proliferation from the former. The third element is the endocardial cushions, proliferation from which forms the upper part.

The commonest defect of the interventricular septum is a small hole at the upper edge of the muscular part due to a deficiency in the component from the proximal bulbar septum, especially easy to imagine where transposition of the great vessels is present   In the present case, however, none of these three elements appears to be present at all

The persistent truncus arteriosus. This is, of course, due to failure of the aortic cushions to form the spiral septum which normally divides off the pulmonary artery from the aorta and as such is a well known abnormality In the present case, it is worthy of note that although the division between the truncus and bulbus arteriosus appears to have been obliterated, the only trace of any of the structures normally derived from either the proximal or distal bulbar septa or even from the cushions which form them, is the tricuspid valve at the orifice of the persistent truncus

The conducting system of the heart Section of the lower part of the wall of the right atrium below the presumptive site of the sino atrial node and of two of the columns of muscle crossing the ventricular cavity failed to reveal the presence of any Purkinje fibres, but it must be noted that the tissue had not been very well fixed in the first instance

I have to thank Sir Arthur Keith and Dr J. Whillis for help in the preparation of this account

THE FAILURE OF WHOOPING COUGH AND ADULT SERA TO NEUTRALISE *PERTUSSIS* TOXIN

D G EVANS

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It is well known that *H pertussis* elaborates a toxin with lethal and dermo necrotic properties and capable of producing a specific antitoxin The significance of *pertussis* toxin in the pathogenesis of whooping cough is not known and although, as previously pointed out (Evans, 1942), it is possible that the toxin may play a part in establishing infection by damaging tissue in advance of invasion by the bacilli, there is not yet sufficient evidence to suggest that antitoxic immunity is of value in prophylaxis

In recent years, reports have been published by Strean (1940), Strean, Lapointe and Dechene (1941) and Kunstler (1945) suggesting that *pertussis* antitoxin is present in the blood of persons with a history of whooping cough, that it can be detected by the intradermal injection of *pertussis* toxin, and that this skin test has the same significance in whooping cough as the Schick test has in diphtheria The results obtained by these workers were not confirmed by Silverthorne *et al* (1944), who found that the skin test with *pertussis* toxin did not distinguish between children who had had whooping cough and those who had not Moreover, Evans and Maitland (1939) had shown that sera from patients with whooping cough, taken up to 9 weeks after the onset of illness, did not contain *pertussis* antitoxin However, in view of the recent work of Strean and others, it was considered advisable to make a further investigation for the presence of *pertussis* antitoxin with a larger number of sera from whooping cough patients and with sera from adults

Sera

Forty sera were obtained from whooping cough patients in hospital , 90 per cent were taken after the sixth week of illness and 40 per cent after the tenth week Seventeen sera from adults were also obtained , 7 of these were from

doctors and nurses in daily contact with whooping-cough, while 3 were from persons who had had the disease in infancy.

Test for antitoxin

Pertussis toxin in the form of an extract from frozen, thawed and ground bacilli (Evans and Maitland, 1937) was used. Two-fold dilutions from 1 : 20 to 1 : 640 were made in saline and control tests showed that 0·1 c.c. of the 1 : 160 dilution produced, on intradermal injection into rabbits, the typical necrotic reaction which has often been described, while the 1 : 320 dilution gave this reaction only occasionally. With each serum sample, 0·15 c.c. of undiluted serum was added to 0·15 c.c. of each dilution of toxin and the mixtures kept at room temperature for 3 hours, when intradermal injections were made of 0·2 c.c. of each mixture into the shaven back of a rabbit. Three or four sera together with the control of toxin alone were usually tested on one rabbit. Some of the sera were also tested on guinea-pigs, but these animals were found to be less suitable for the test since they were not as susceptible to the skin reaction as rabbits. Six of the sera from adults were also tested for neutralising antibodies to lethal doses of toxin in mice. With the toxin employed, 0·1 c.c. of a 1 : 4 dilution always proved fatal for 18-20 g. mice when given intravenously, while a dilution of 1 : 8 was occasionally fatal. Four dilutions of toxin, 1 : 2, 1 : 4, 1 : 8 and 1 : 16, were used and to 0·1 c.c. of each dilution 0·4 c.c. of undiluted serum was added, the total mixture of 0·5 c.c. being injected after remaining at room temperature for 3 hours. A control titration was made with 0·1 c.c. of each dilution of toxin mixed with 0·4 c.c. of saline.

Results

None of the 57 sera, when tested by the intradermal method, contained *pertussis* antitoxin sufficient to neutralise the smallest dose of *pertussis* toxin which produced a skin reaction. With each serum there was no difference between either the skin reaction caused by the injection of mixtures of toxin and serum or by toxin alone. Neither was there any neutralising property shown by 6 of these sera when tested by the mouse intravenous method.

Summary

Forty sera from cases of whooping cough and 17 from adults were found to contain no *pertussis* antitoxin.

This result does not support the suggestion made by Strean and his collaborators that persons with a history of whooping cough possess circulating *pertussis* antitoxin which can be detected by the intradermal injection of *pertussis* toxin.

I am indebted to Dr E. H. R. Harries, Medical Superintendent of the London County Council North Eastern Hospital, and Dr Joyce Wright for the majority of sera used in this investigation.

REFERENCES

EVANS, D. G. . . . . . . 1942. *Lancet*, i, 529.
EVANS, D. G., AND MAITLAND, 1937. This *Journal*, xlv, 715.
  H. B.
  „   „   „   „   1939. This *Journal*, xlviii, 465.
KUNSTLER, M. . . . . . . 1945. *Canad. Med. Assoc. J.*, lii, 62.
SILVERTHORNE, N., FRASER, D. T., 1944. *Ibid.*, l, 129.
  AND BROWN, A.
STREAN, L. P. . . . . . . 1940. *Ibid.*, xlii, 525.
STREAN, L. P., LAPOINTE, D., AND 1941. *Ibid.*, xlv, 326.
  DECHENE, E.

BOOKS RECEIVED

Human torulosis

By 'LEONARD B. COX and JEAN C. TOLHURST. 1946. Melbourne and London : Melbourne University Press in association with Oxford University Press. Pp. xi and 149 ; 67 figs. on 15 plates. 25s.

This Australian monograph on a rare but insufficiently recognised disease is both comprehensive and concise. It opens with a brief outline of the history of the disease, which formerly was often confused with blastomycosis. The authors then give a detailed account of 12 personally studied cases, with the necropsy findings in 8—the largest series of cases yet reported from a single source. There follow well documented reviews of the clinical aspects of the disease, the properties of *Torula histolytica* and its pathogenicity for animals, the pathology of the lesions in man and animals, and the laboratory diagnosis of torulosis. In all of these the authors succeed in combining a well-balanced account of previous findings with the fruits of their own extensive experience. Indeed the first merit of the work is that it is a record of personal research and not a mere compilation. This applies particularly to the sections on microbiology and pathology, which bear witness to much careful and original laboratory investigation. Consequently the monograph, while valuable to the clinician, will be of even greater value to the pathologist and bacteriologist. The 67 photographs, most of which depict the gross and microscopic appearances of the lesions and the morphology of the organism in the lesions and in culture, are clear and well chosen. Finally, there are a comprehensive list of 134 references, an adequate index, and an appendix necessitated by war-time delay in publication in which the authors add their thirteenth case and bring their review of the literature up to date.

This is a wholly commendable monograph ; it should stimulate interest in a disease which is evidently not the great rarity many have supposed, which can easily escape diagnosis unless deliberately searched for, and of which, be it noted, very few cases have so far been reported in Great Britain.

Experimental hypertension

By WILLIAM GOLDRING *et al.* 1946. New York. Being volume III of the special publications of the New York Academy of Sciences. Pp. x and 179 ; 45 figs. $3.75.

In February, 1945, the New York Academy of Sciences held a symposium on experimental hypertension in which prominent American investigators read papers on many aspects of the subject and discussed their results. Dr Harry Goldblatt gave the introductory lecture in which he outlined with great clarity the events leading up to his significant experiment with the Goldblatt clamp. It will be remembered that the outcome of this method was the demonstration that hypertension develops in the dog after constriction of the main artery of one kidney. Subsequent experiments showed that the same phenomenon can be induced in a host of animals and that in some of these, especially the rat and sheep, the blood pressure

remains high for many months, whereas in others the hypertension disappears in a few weeks. A prompt recovery follows release of the obstruction or removal of the kidney with the constricted renal artery. Despite constriction of both main arteries in the dog—a procedure which leads to persistent hypertension—no accompanying upset of renal excretory function can be detected ; hence the conclusion that there is a type of hypertension of renal origin without gross disturbance of excretory function. On the other hand, when renal function becomes impaired, fatal uræmia results and autopsy invariably reveals pathological changes in the vessels of the kidney and other organs reminiscent of the malignant phase of essential hypertension in man.

It is not proposed to describe at length the outcome of these important results, for the ramifications of the subject are many, but reference to the general conclusions reached at the New York symposium may be enough to persuade the reader interested in hypertension to study this important compilation.

Most of the speakers agreed that interference with the blood flow through the kidneys initiates a mechanism which leads to some form of hypertension. This is independent of nervous factors and most likely humoral. Much evidence exists in support of a *circulating pressor substance* (hypertensin or angiotonin) formed through the action of renin—a proteo-lytic enzyme normally reposing in the kidneys—on a blood globulin variously known as renin substrate or hypertensinogen. Interference with renal hæmodynamics results in the release or formation of abnormal amounts of renin. No other tissue, apparently, can produce this compound, since a rise in blood pressure does not follow impairment of the blood supply of the spleen, the liver or the parts of the body supplied by the aorta below the origin of the renal arteries. However, it is only fair to say that alternative suggestions have been made and some workers maintain that in such circumstances there is decreased formation of an anti-pressor substance normally found in the kidneys. A further opinion is that angiotonin exerts a pressor effect only when another (as yet unknown) substance is present in the blood. Indeed, the problem is complicated by the lack of convincing proof that angiotonin is the cause of the hyper-tension and a further factor may ultimately prove to be involved in the mechanism.

To make things still more complicated a protein component of kidney extract with an anti-pressor action has been identified and is presumed to contain an angiotonase or hypertensinase, but no proof has yet been advanced that this, as such, enters the circulation after its injection. An anti-renin substance, too, can be developed in animals receiving repeated intramuscular injections of renal extracts containing renin, and in hyper-tensive animals this gives a fall in the blood pressure.

Although much useful information about hypertension has been derived from these experiments it seems that little has been contributed so far to the human problem, for it is not yet certain that the primary disturbance in the experimental animal and in man are identical. Anatomical evidence of disturbed renal flow is far from constant ; indeed, the general impression favours the view that the renal circulatory alteration is a secondary effect resulting from the action of an unknown pressor agent. Clear evidence of a *renal type of hypertension in man probably exists in no more than 10 per cent. of the cases* in adults and we should be cautious about applying conclusions drawn from such a small minority to the generality of cases. No better summary of the present position can be given than by quoting the words of Drs. Goldring, Chasis and Smith (page 178): "Human hypertensive disease is a complex disorder in which elevated blood pressure

is merely one manifestation The production of elevated blood pressure in the experimental animal cannot be construed as evidence of the production of hypertensive disease, nor can the assumption be accepted that, when the blood pressure is elevated, the animal has the counterpart of the human disease, nor that, when the blood pressure is lowered to normal levels by presumably specific antipressor agents, the animal is cured of the disease " A gloomy though salutary corrective[1]

Marine microbiology

By CLAUDE E ZoBELL 1946 Waltham, Mass , The Chronica Botanica Co London , William Dawson and Sons Ltd Pp xvi and 240 , 12 text figs $5

This book reviews and summarises an extensive literature on marine bacteria and other micro organisms as biochemical, geological and hydro biological agents A series of chapters deals with the marine environment, the distribution and characters of the marine bacteria, the micro organisms of the sea bottom and their activities and their sanitary and economic aspects The author has brought together a large amount of information scattered widely in the literature and has knit the material into a well documented and coherent whole In the sections dealing with the collection and examination of samples taken at sea, methods of enumeration, studies on distribution and general nature of the flora of the sea bottom—subjects in which the author and his collaborators have carried out pioneer work—the relevant data are admirably presented It is in fact the best critical exposition of these subjects which the reviewer has met and should be read with advantage by most bacteriologists The chapters on the sanitary and economic aspects of marine microbiology are not up to the same level There is confusion in the section on the bacteria present on sea fish For example, data for newly caught fish are given without comment alongside those from fish in which no precautions were taken to exclude contamination from human sources The autochthonous bacterial flora of fish freshly caught and placed in sterile containers is vastly different qualitatively and quantitatively from that of fish which have been handled by man and examined after storage in ice, which itself contains numerous bacteria The organisms of the surface slime and of the intestinal contents rapidly penetrate the vascular gills and quickly invade the tissues by the blood stream In this connection, the excellent work of Lucke and Schwartz giving quantitative data of viable organisms from the living fish till it reaches the retailer is omitted, although mention is made of this work in general terms in the section on spoilage of marine products As in so many American books, contributions from European sources are almost ignored

The inclusion of the halophiles which cause the reddening of salted fish along with the bacteria of marine fish cannot be justified In another section the halophiles are treated as derived from solar salts and no cross reference is given The statement on p 190, quoted from Beckwith, that *Diplococcus gadidarum* can be isolated from discoloured unsalted codfish, given just before mention of the discolouration of unsalted halibut, would cause confusion to anyone unfamiliar with the halophiles This organism is present only on salted fish The inclusion of *Clathrocystis roseo persicina, Oidium fulvunatum* and *Torula epizoa* as causal agents of reddening of salt fish is not in accordance with modern work

The section on the bacteriology of ice is inadequate The author has depended on Jensen's circumscribed review. European data on the subject have been largely ignored and nothing is said of artificial ice Of the quite

voluminous literature on germicidal ices only two papers, are mentioned (one Canadian and one American). In the treatment of the characteristics of marine bacteria and the transformation of organic matter several points arise. The treatment of the luminous bacteria and of their voluminous literature is quite inadequate. Further, the classification of Achromobacter, Flavobacterium and Micrococcus will remain difficult until more is known of the biochemistry and variability of these organisms.

Of more general interest is the statement on p. 142 that proteolytic bacteria are primarily responsible for the spoilage of fish, shellfish, crabmeat and other marine foods. This is too sweeping. It is now known that spoilage of fish, stored in ice, is detectable several days before there is obvious breakdown of protein. Trimethylamine is produced from trimethylamine oxide, not from fish protein as ZoBell seems to infer from the statement on p. 143. It appears several days before the ammonia which arises from deamination of the protein.

There is much speculation on the relation of bacteria to geological formations, based on very general findings, but there is necessarily a lack of specificity with regard to the characters of the bacteria.

The anaerobes of special interest in the mud of the sea bottom are no more than barely mentioned but great importance is attributed to them in the chemistry of the sea bottom.

This is a book which bacteriologists would do well to read. To students of marine microbiology it is indispensable.

There is a bibliography of 676 items.

Chemotherapy

By Sir ALEXANDER FLEMING. 1946. London : Cambridge University Press. Pp. 39 ; 5 text figs. and 2 plates. 2s.

This Linacre lecture is a concise review of one branch of a very wide subject, being limited to chemotherapy of bacterial infections. The first part is devoted to a consideration of local chemotherapy, starting with Lister's use of carbolic acid as an antiseptic and passing in review the various chemical agents subsequently introduced for the treatment of wounds. The importance of the relative toxicity of chemotherapeutic substances to leucocytes and bacteria is stressed, and is illustrated by the author's own observations on formalin, quinine, eusol and mercuric chloride, using the slide-cell technique. With the exception of Ehrlich's salvarsan, however, all the chemicals tested for systemic therapeutic activity against bacterial infections failed until the introduction of the sulphonamides. The merits and limitations of the sulphonamides are discussed and relatively little space is given to a consideration of the unique properties of penicillin. The potentialities of gramicidin, streptomycin and other antibiotics are briefly indicated. In relation to the future development of chemotherapy in Britain, the need for a central institute for fundamental research in microbiology is stressed.

A textbook of bacteriology and immunology

By JOSEPH M. DOUGHERTY and ARTHUR J. LAMBERTI. 1946. London : Henry Kimpton. Pp. 360 ; 102 text figs. 22s. 6d.

This book, being written primarily for the pre-medical or pre-dental stage of the student's curriculum, makes little reference to the importance and functions of bacteria in the economic sphere, whilst the pathogenic organisms are dealt with in some detail. Suitable emphasis is given to elementary optics and the fundamentals of microscopy, but a diagram

such as fig 11, of eight lines, is only misleading, since (*a*) the focal point
of the condenser is below the upper surface of the slide, (*b*) the ray emerging
at the glass air interface is not refracted, (*c*) the convergent pencil of light
from the condenser is asymmetrical, and (*d*) the boundary of the oil droplet
is concave to the right instead of to the left

After a brief section dealing with yeasts and moulds, a well illustrated
section on bacteriological apparatus, culture media and manipulative tech
nique follows   This is marred by inaccuracies, for example on page 72
lævulose is described as inverted cane sugar, whilst galactose is derived
from the hydrolysis of starch   It seems inappropriate to suggest the use
of mnemonics to the student's mind (p 47) and it is certainly misleading
to state that ' Bacteria reproduce by the budding of spores and by binary
fission ', or, in the light of our knowledge of anaerobiosis, to describe
oxygen (p 63) as " an inorganic constituent of the atmosphere, an absolute
requirement for all living things "

A section on immunology follows in which routine serological tests are
elaborated in some detail, and in a subsequent chapter hæmatological
techniques are briefly described   There are many errors and misprints,
e g an 85 per cent solution of sodium chloride is given as the suspending
medium for sheep erythrocytes, and the area of the hæmacytometer squares
is miscalculated as 1/4000 sq mm

The remainder of the volume deals with the main groups of pathogenic
organisms, including the protozoa and filterable viruses, and with the
bacteriology of water, milk and other foods   The illustrations in this
systematic survey are exceedingly crude drawings   Fig 73, which represents
Mycobacterium lepræ, is particularly puerile, while the legends give no
indication of the magnifications employed   The nomenclature is in
consistent, and misleading for the novice for whom the book is primarily
intended

Atlas of histopathology of the skin

By G H Pircival, A Murray Drennan and T C Dodds   1947
Edinburgh   E & S Livingstone   Pp viii and 494, 370 photomicrographs
in colour   75s

The core of this work is an excellent series of coloured plates depicting
the histological appearances of skin diseases and the authors are to be
congratulated   It will be of great value to the postgraduate student in
dermatology for whom it is intended   The work must be accepted for what
it is, an atlas of histopathology of the skin, to be used in conjunction with
textbooks of skin diseases and textbooks of pathology   The text is
deliberately brief and relatively unimportant, the legends attached to the
plates are simple and descriptive—perhaps a little too simple at times
The reproductions are, almost without exception, excellent, though a few
are too heavily stained with consequent loss of detail   We would have
welcomed more high power reproductions to bring out such detail, but
there is, nevertheless, a wealth of material

One criticism we would offer is that in many of the plates the field
presented is too small, so that the disease depicted is not seen in relation to
and differentiated from the normal tissue with which it might be compared
An example is the single low power section of mycosis fungoides, which
is also one of the few specimens not in focus

The authors attempt in the introduction to justify an unusual arrange
ment of the material presented on morphological grounds but we do not
think this will have a general appeal   Inflammation is presented through
the medium of illustrations of urticaria—including urticaria pigmentosa—

psoriasis, lichen, eczema, prurigo and vesico-bullous eruptions, before impetigo and staphylococcal infections are dealt with.

There is some disproportion, which should be corrected in subsequent editions. Thus psoriasis, varicella and vaccinia are each given four illustrations while keratodermia blenorrhagica has five and the pigmented nævus twenty-seven. Much attention is given to the formation of vesicles and bullæ, and the thesis is put forward that in eczema this is not the result of filtration from papillary œdema.

Not all dermatologists will subscribe to the general conception of eczema put forward. This is inevitable, but with all respect to the views of the Edinburgh school, we think it unfortunate that seborrhœic dermatitis and other commonly accepted seborrhœic disorders should not be mentioned as such, if only in brackets.

It is unfortunate too that, after a series of good plates of the normal histology, the work should open with such rare affections as pseudo-xanthoma elasticum, amyloid infiltration, sclerodermia, calcinosis, dermato-myositis and necrobiosis lipoidica. Five illustrations are assigned to the latter affection, which is, we think improperly, related by the authors to granuloma annulare. On the other hand no mention is made of the rheumatoid nodule which, histologically, may be identical with granuloma annulare. Another omission is the illustration of factors concerned in pigmentation of the skin. We suggest that it would enhance the value of the plates if there were, in addition, clear large-scale examples, perhaps diagrammatic, of the cells composing various tissues and taking part in different reactions.

This work is, nevertheless, a valuable addition to the library of the dermatologist and the histopathologist.

Skin diseases, nutrition and metabolism

By ERICH URBACH, with the collaboration of EDWARD B. LeWINN. 1946. London : William Heinemann (Medical Books) Ltd. Pp. xxii and 634; 266 text figs. 50s.

Dr Urbach has attempted a difficult task with some success. He has put much labour into the work, and presents a well-illustrated volume of pleasing appearance. The historical introduction is interesting and the general approach to the subject good. The value of the biochemical investigation of the skin as opposed to the blood is stressed, but little work has been done in this field except by the author himself.

The informed reader will appreciate the collection in one volume of much of what is known and accepted of the metabolism and biochemistry of the skin and of the influence of diet. It is useful also to have some particulars of alleged metabolic causes of skin diseases, but the uninformed reader will find much of this confusing, for weighty authority and evidence are often brought to support entirely opposing points of view and no very critical evaluation is offered. The chapter on psoriasis is an example of this and in general too much is made of biochemical findings which, in relation to most constitutional and allergic reactions, are probably incidental and of no basic significance.

The reviewer would have liked a more adequate consideration of the disturbances of carbohydrate and fat metabolism, general and local, and of the influence and character of disturbances of the sodium, potassium and calcium balance, which receive no attention. In a work of these proportions some attention might have been given to such affections as myxœdema, dermatomyositis, sclerema and sclerodermia. In this connection Urbach incorrectly reports Sellei as regarding acrosclerosis as identical

with sclerodactylia   in fact Sellei is responsible for the clear differentiation of these two affections   The clinician will question some of Dr Urbach's assertions and will not easily accept at their face value some of the illustrations indicating response to the dietetic treatment of neurodermatitis, psoriasis and other constitutional dermatoses   There is much in the text that could with advantage be cut, while subjects like tuberculosis, perniosis and eczema demand a broader and more balanced approach   The reader is left with the feeling that there is an attempt to record all that has been said and written on the subject instead of selecting the profitable work and presenting this to more purpose

Fundamentals of immunology

By W C BOYD   Revised reprint, 1946   New York and Toronto, Staples Press Ltd   London   John Bale Medical Publications Ltd   Pp xiv and 446, 45 text figs   33s

The author has been remarkably successful in presenting the fundamentals of immunology for the beginner and at the same time offering information and discussion useful to the " professional immunologist "

The opening sentences of the book forecast a limited treatment   " What we are really interested in is degrees of *resistance* to disease   "   But in chapter VIII, on anaphylaxis and allergy, the " unwary reader " is told that he must broaden his conception of immunology and interest himself in a general way in the mechanisms brought into action on exposure to a specific foreign substance   This evolution is characteristic of the book, which bears throughout the mark of a skilful and patient teacher prepared to approach difficulties in the most gradual fashion yet determined that his pupil shall not avoid them

Modern conceptions are stated first and much contradiction and confusion are avoided by including historical information only if it is relevant   The bibliography is extensive but it discriminates fairly and wisely and there are 86 useful pages on laboratory and clinical technique

Interpretation of the Widal reaction in the diagnosis of typhoid has been summarised (p 361) " rather roughly '—so roughly that the guidance offered is seriously misleading on some points   For example   " The O may be positive and the H negative   This means the disease is not typhoid, or if typhoid, too recent for the H agglutinins to have developed

And again   " The O may be negative and the H positive   This means the disease is not now active, or the patient has previously been vaccinated against typhoid "

These errors require correction in the next edition but they are not representative and do not seriously diminish the value of the book, which may be recommended with confidence to both students and lecturers

The bacterial cell in its relation to problems of virulence, immunity and chemotherapy

By RENÉ J DUBOS   Second printing, 1946   Cambridge Mass   Harvard University Press (London   Geoffrey Cumberlege)   Pp xix and 460, 22 text figs and 20 plates   28s

A second printing of this authoritative and informative work has been called for within a year of publication, a striking tribute to its great merits (For review, see this *Journal*, 1946, lviii 509)

An introduction to bacteriological chemistry

By C. G. ANDERSON. Second edition, 1946. Edinburgh: E. & S. Livingstone. Pp. x and 500 ; 11 text figs. 20s.

In his new edition the author has provided a chapter on bacterial growth factors which comprises a great deal of useful information within a small compass, including a well-arranged table of the vitamin requirements of a number of organisms. This chapter is up to date and valuable. The same is true of the new chapters X and XI, which introduce the student to modern aspects of chemotherapy and antibiotics respectively. These three additions greatly enhance the value of the book. In chapters XXII and XXIII the student is ably introduced to the chemical aspects of immunity, whilst chapter XVI deals with the fermentations and metabolic products of the lower fungi (moulds) and comprises a lot of information in a small space, usefully introducing a subject not often presented to elementary students.

The first four chapters are devoted to general elementary biochemistry, which the student could obtain better from an elementary textbook of the subject. The space so saved would have enabled the author to deal more adequately with his crowded programme and perhaps to give more space to bacterial photosynthesis and to include a more adequate account of filterable toxins.

On a completely different level are chapters XIV and XV which deal with alcoholic and bacterial fermentation. It is almost incredible at this period to see the schemes of Neuberg (1919-1925) for alcoholic fermentation and the provisional scheme of Kluyver (1931) for bacterial fermentations— useful as they were when put forward—treated as if they were still of an importance and validity comparable with the modern Embden-Meyerhof-Parnas scheme, every step of which has now been experimentally proved and every intermediate and almost every enzyme involved isolated. It also grossly misrepresents the views of Neuberg and of Kluyver, who have never claimed that their respective schemes are still tenable in view of modern work. During the last six years bacterial fermentations have been shown to conform to the same fundamental plan as alcoholic fermentation, with additional products due to different enzyme systems. This valuable and masterly work, coming chiefly from the Iowa school, has been almost completely ignored and the student instead fobbed off with obsolete hypotheses in which acetaldehyde and methyl glyoxal hold key positions.

It appears from internal evidence and the list of recommended reading that the author has here (as also in the chapters on nitrogen metabolism and bacterial respiration) relied on obsolete textbook material instead of on original papers or even on the many excellent reviews appearing annually. So little interest does he take in these aspects of his subject that he puts forward contradictory theories for the same process in different sections, as, for example, when the formation of methane is attributed to CO_2 reduction on p. 76 and to cellulose fermentation in the chapter on industrial fermentation.

Synopsis of pathology

By W. A. D. ANDERSON. Second edition, 1946. London : Henry Kimpton. Pp. 741 ; 327 text figs. and 14 colour plates. 32s. 6d.

The new edition of Anderson's " Synopsis " contains 80 more pages than the first ; but by the use of a lighter paper, both bulk and weight have been reduced. Of the 327 text figures, 74 are new, mainly by

substitution Revision of the text has been extensive, and greater emphasis has been given to tropical diseases and conditions important in "war medicine" The chapters dealing with viral, rickettsial, spirochetal, mycotic, protozoal and helminthic infections have been enlarged and made more inclusive and some new subjects like epidemic hepatitis and blast injuries have been given attention Chapter VI, for example, on rickettsial and viral diseases (both written and revised by Dr Henry Pinkerton) has grown from 17 to 33 pages, and the bibliography from 10 to 46 items This well balanced chapter provides just the right kind and amount of information for the undergraduate concerning a group of diseases of which he cannot be expected to have much first hand knowledge but about which he may well be expected to answer questions in an examination

The section on worm infections—quite inadequate, even for examination purposes, in the first edition—has been enlarged from 4 to 9 pages, and now includes a new 2 page account of schistosomiasis A number of important points of detail are however still omitted, or dealt with only very cursorily The pathological effects of hookworm infestation, for example, are still given in two lines (" the condition is often accompanied by severe anæmia, eosinophilia, and evidence of general intoxication "), and there is still not a word about the pathogenic effects of *Diphyllobothrium latum* or its geographical distribution, surely a subject of special interest and importance to Americans

In the section on asbestosis (p 370), the statement continues to be made that " Inhalation (of asbestos fibres) over a period of seven or more years is usually required to produce the disease " Surely it is a matter of the amount of dust inhaled In the past, prior to the introduction of safety devices, many workers in very dusty atmospheres have inhaled enough dust in 6 months or a year to produce, at a later date, severe asbestosis from which they died This is a point to which we drew attention in reviewing the first edition (this *Journal*, 1943, iv, 384) Nor can the statement that the pulmonary fibrosis is due to mechanical rather than specific chemical action be allowed to pass unchallenged It is contrary to the best modern opinion The basal distribution of the fibrosis should be mentioned The brief note on the sidero silicosis of hæmatite miners conveys a wrong impression of the ætiology of this condition It reads " Silico siderosis is due to inhalation of iron containing hæmatite by hæmatite miners The lungs have a striking brick red colour Some silica is usually inhaled as well A diffuse or nodular pulmonary fibrosis may be produced " The reader would hardly gather from this, (a) that hæmatite invariably contains a substantial proportion of silica, (b) that it is this silica and not the ferric oxide which is the noxious agent

Taken as a whole, however, Dr Anderson's little book represents a praiseworthy attempt to provide the student of medicine with a synoptic account, well illustrated, of pathological processes, with emphasis on the morbid anatomy and histology For examination and revision purposes it should be particularly useful The serviceable lists of references, mainly American, have been brought well up to date

Autopsy diagnosis and technic

By OTTO SAPHIR Second edition, 1946 New York and London Paul B Hoeber Pp xviii and 405 , 69 text figs $5

We have not seen the first edition of this manual The preface to the second edition mentions the inclusion of additional material on diseases of the breast, accidental death, stillborn and newborn infants, the nose and accessory sinuses, vitamin deficiencies, and certain tropical diseases

The author disclaims all intention of producing a text-book of pathology. The two prefaces and Dr Ludwig Hektoen's foreword permit the inference that the book is not for pathologists so much as for students and clinicians, whose capacity to take advantage of the opportunities of autopsies it hopes to increase. This object may well have been achieved.

The scheme adopted is to give in detail one method of making a post-mortem examination, and to introduce descriptions of the morbid appearances which may be found in each organ as it comes under examination. Dr Saphir favours the removal of the thoracic, abdominal and pelvic contents *en masse*. It happens that the reviewer does not share this view, but pathologists like everybody else are creatures of habit, and probably find most convenient the methods to which they have become accustomed. The important thing is that Saphir offers a practicable plan of attack on his visceral mass, and a lucid explanation of the approach to each individual organ. One feels that an adequate autopsy, from the operative point of view, could be carried out by a beginner armed with this book and plenty of time.

The descriptive morbid anatomy is on the whole sound and clearly expressed, but there are a few unaccountable lapses, *e.g.* it is suggested (p. 94) that infarction of the lungs commonly follows embolism resulting from systemic venous thrombosis, though later (p. 187) a more orthodox statement on pulmonary infarction is given. There has evidently been a slip in fig. 20, which shows the adrenal and sympathetic ganglia (but not the kidney) as sites of hypernephroma, because the correct version is given in the text (p. 121). The short paragraph on p. 191 is an insufficient guide to the differentiation between lobar pneumonia and confluent broncho-pneumonia. One wonders if the author has had any personal experience of pneumoconiosis when he gives a single composite eight-line description for anthracosis, silicosis, siderosis and asbestosis. The word arteriosclerosis has lost any precise meaning ; in this book it is used for atheroma (p. 228). Osteoma seems a formidable designation for the heterotopic bony fragments found in the spinal meninges (p. 326). In chronic passive hyperæmia, *pace* p. 275, the spleen is not necessarily enlarged, its capsule and trabeculæ are not thickened, and the follicles are usually clearly visible.

On the whole this book should realise its author's purpose. It is easy reading, and has the great merit—to this reviewer at any rate—of being printed on matt paper.

Clinical laboratory diagnosis

By SAMUEL A. LEVINSON and ROBERT P. MACFATE. Third edition. 1946. London : Henry Kimpton. Pp. 971 ; 192 text figs. and 15 plates (7 in colour). 50s.

The general plan of this work remains the same as in the first and second editions (reviewed in this *Journal* : 1937, xlv, 786 ; and 1943, lv, 510). The authors' preface states that data and procedures have been brought up to date, chemical methods " critically revised to simplify each detailed step ", and some new features added. Of the last named the most important is a chapter on tropical medicine, which is relatively satisfactory, probably because its subject is better adapted than most to the manner of treatment.

The strongest feature in the book is the description of technical procedures in given examinations and especially in chemical examinations. In a large proportion of cases these are set out with a clarity and precision of detail which ought to make them possible even to the veriest tyro. But the indications of when and why these examinations are to be done are on a

much less satisfactory level, and the alphabetical list of diseases (pp. 923-933) with their appropriate laboratory tests—and the page numbers of the latter— seems to condone something approaching penny-in-the-slot pathology.

The worst section is that on bacteriology. The space allotted is quite inadequate and much of the information in the tables (pp. 634 *et seq.*) is antiquated and useless from a practical point of view. Insufficient attention is given to serological methods in the identification of fæcal bacteria, and the brief notes on the investigation of diphtheria would be useless alike to novice and expert. The method of blood culture put forward seems to combine a substantial risk of contamination with a considerable chance of failing to grow the pertinent organism.

It is still impossible to commend this book. In one respect it may even be dangerous, through enabling clinical pathological investigations to be carried out and "interpreted" by those who have not a proper grasp of their implications.

Principles of hematology

By Russell L. Haden. Third edition. 1946. London : Henry Kimpton. Pp. 366 ; 171 text figs. and 1 colour plate. 25s.

This small book on hæmatology has now reached its third edition. The author's purpose is " to keep this volume a simple discussion of the fundamental principles of hematology ". As such, therefore, it is written " for the student and physician ". It obviously does not meet the requirements of the pathologist, as the information given amounts to little more than the bare bones of blood cytology and of the standard estimations and calculations. Illustrative case records fill about one third of the book, photographs and diagrams a further quarter. This leaves little room for informative text and, indeed, what text remains is so repetitive as to make its reading tedious. Moreover, tables, diagrams and legends to figures are often used to repeat what has already been said more than once in the text.

The principles elaborated are those which virtually limit hæmatology to a study of the peripheral blood as the *via media* between bone marrow and tissues. This mechanistic conception is emphasised in numerous diagrams of the " popular economics " type. The broader pathological and clinical view tends to be obscured by too much dependence on simple illustration and example. The student, having been led through a brief review and classification of leukæmia (including the doubtful chronic monocytic type) is presented with case records of seven cases which are certainly of interest, but he will fail to gain any idea of the natural history of the disease as a whole, its morbidity and mortality or even of the highly important age incidence of the common types.

The method of selection of material for this book is difficult to understand. The author includes technical instructions for estimation of iron equivalents, prothrombin time and heterophile antibodies, and intentionally leaves out not only the technique of Rh testing but all mention of the role of the Rh factor in disease. He has now included a short section on marrow biopsy, thus rectifying one of the grosser omissions pointed out in this *Journal's* review of the previous edition, but he has not brought the book up-to-date. No reference is made to published work later than 1939 and much of his discussion is based on premises recently modified, such as the identity of the megaloblast and the survival period of the erythrocyte.

The book is well illustrated and pleasingly produced, though not free from misprints, and will probably maintain currency among those clinicians who like to keep their hæmatological conceptions stable and simple.

J. F. HEGGIE and IVOR LEWIS.   (1) Sarcoma of bronchus (treated by radiation) :
    biopsy sections.   (2) Carcinoma of lung : biopsy and gross (pneumonectomy)
    specimens.   (3) Carcinoma of head of pancreas (Whipple operation) : excised
    specimen.
MARTIN BODIAN.   Eosinophilic granuloma of bone.

CINEMATOGRAPH FILM

The cultivation of tissue *in vitro* : the maintenance of living tissue
outside the maternal organism

Abstracts

576 . 8 . 095 . 4 . 576 : 851 . 49 (*B. dysenteriæ*) 616 . 33—008 . 8

OBSERVATIONS ON THE SURVIVAL OF *B. DYSENTERIÆ*
IN GASTRIC JUICES

J. C. DICK

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Royal Infirmary, Glasgow*

The following experiment was carried out in a few cases of bacillary dysentery.
When the patient was convalescent, a fractional test-meal was performed and
the free and total acidity measured in the usual way.   Thereafter, to 0·75 ml.
of each fraction (filtered through gauze) 0·25 ml. of a 100 million per ml.
suspension of a 24-hour agar culture of the patient's own infecting organism
was added.   After thorough mixing and incubation at 37° C. subcultures were
made every quarter of an hour for one hour and again at two hours.   The
survival rate of the organisms in each fraction was compared with the acidity
of that fraction.   Simultaneously, the above amounts of the suspension of
organisms were mixed with various dilutions of hydrochloric acid in distilled
water and the survival rate tested in the same way.

In the first case tested, the organisms (*B. dysenteriæ* Flexner) survived up
to 2 hours in four fractions, all of which showed no free hydrochloric acid,
though one of the fractions had a total acidity of 0·073 per cent. before dilution
with the suspension.   In two fractions (½ hr. and ¾ hr.) where there was
0·073 per cent. free HCl with higher total acidity, the organisms did not survive
for 15 minutes (really 8 minutes at 37° C., as 6 or 7 minutes were taken up
in mixing all the fraction and suspension mixtures).   Tested in dilute HCl
only, this strain of the dysentery bacillus survived in 0·05 per cent. for 15
minutes but not for half an hour, and not even for 15 minutes in
0·075 per cent. acid.

In another case of Flexner dysentery, a lethal concentration of HCl was not
reached until the one-hour fraction.

A case of Sonne dysentery showed a high curve for free HCl but, as often
occurs early in such curves, the ¼ and ½ hr. fractions showed a free acid of less
than 0·036 per cent. and the organisms survived in them for at least 1½ hours.
In aqueous dilutions of HCl the bacilli survived for 15 minutes in 0·05 per cent.

In the fourth test, another Sonne dysentery, there was a low level of free
HCl throughout, only one fraction (with 0·073 per cent. HCl) killing the organisms
within 15 minutes, although the total acidity reached 0·182 per cent. in another
specimen.

In a case with Shiga dysentery, the results were similar

Thus the survival of the organisms in the fractions depended upon their percentage content of free hydrochloric acid

Garrod (1937) showed the varying susceptibilities of various intestinal pathogenic organisms to the bactericidal action of gastric juice or its active constituents   *Br. abortus* and *Br melitensis* were most susceptible, then *Bact typhosum*, while *Sh flexneri* was least susceptible of those tested   His observations support the proposition that the infectivity of intestinal pathogens varies with their resistance to bactericidal action in the stomach

Wilson and Miles (Topley and Wilson, 1946) state that the empty stomach is generally sterile, but that immediately after a meal it contains numerous organisms (ingested with the food), most of which appear to be killed off rapidly They go on to state that, if the motility of the stomach is excessive or the acidity below normal, this sterilising effect of the gastric juice is probably incomplete

Samson Wright (1945) points out that the peristaltic waves in the wall of the stomach are normally gentle and not very deep   there is no general vigorous churning up of the food with the gastric secretion   Also, after 5 15 minutes from the taking of food, the pyloric sphincter relaxes from time to time to permit a small amount of chyme to escape   Thus any organisms ingested with the food may be exposed to the action of the gastric juice for only 5 minutes In the test meals performed in this experiment, there were fractions, especially in the early stages, where there was not sufficient free HCl to kill the dysentery bacilli

Thus the bactericidal effect of the gastric juice appears to depend on the presence of a certain concentration of free HCl, varying even for different strains of the same organism   More important factors in allowing or preventing infection by intestinal pathogens are the position of the organisms in the meal and the rate of their passage through the stomach, as these points will determine whether the gastric juice has an opportunity to exert its bactericidal effect or not

REFERENCES

GARROD, L P .	1937	This *Journal*, xlv, 473
TOPLEY, W W C , AND WILSON, G S	1946	Principles of bacteriology and immunity 3rd ed , revised by G S Wilson and A A Miles, *London*, vol ii, p 1987
WRIGHT, S	1945	Applied physiology, 8th ed , *London*, p 754

576 8.093 4

A METHOD FOR STAINING BOTH GRAM POSITIVE AND GRAM NEGATIVE BACTERIA IN SECTIONS

W S OLLETT

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In our hands the methods so far published for staining both Gram positive and Gram negative bacteria in sections have given very unsatisfactory results Sections stained by Glynn's method (1935) and by Goodpasture s method as modified by MacCullum (McClung, 1937) showed inadequate staining of the bacteria and poor contrast with the background   The Gram Pappenheim method for Neisseria devised by Sandiford (1937) gave fair contrast with the background,

but the staining of the Gram-negative bacteria was faint, and that of the Gram-positive bacteria indifferent and patchy.

We have found that the following modification of the Twort method (Twort, 1924) is easy to carry out and gives consistently excellent contrast between Gram-positive bacteria, Gram-negative bacteria and tissues. The light green in Twort's stain is replaced by fast green F.C.F. to avoid fading.

METHOD

Fix material in 5 per cent. formol-saline, pass through the alcohols and embed in paraffin.

Cut sections at 3 μ.

Remove the paraffin in the usual way and bring down to distilled water.

Gram-Twort stain

1. Stain in aniline-crystal violet, 3-5 minutes.
2. Pour off stain and wash quickly in distilled water.
3. Treat with Gram's iodine, 3 minutes.
4. Pour off the iodine, wash quickly in distilled water and blot dry.
5. Decolourise with 2 per cent. acetic acid in absolute alcohol until no more colour comes away : the section should be a dirty straw colour at this stage. (This differentiator is much easier to control than aniline-xylol.)
6. Wash quickly in distilled water.
7. Counterstain in Twort's neutral red-fast green stain, diluted 1 part with 3 parts of distilled water, for 5 minutes.
8. Wash quickly in distilled water.
9. Decolourise with 2 per cent. acetic acid-alcohol until no more red stain (neutral red) comes away (15-30 seconds).
10. Clear in xylol and mount in D.P.X. or neutral balsam.

RESULTS

Nuclei red ; cytoplasm light green ; red blood corpuscles green ; Gram-positive bacteria dark blue ; Gram-negative bacteria pink.

I wish to thank Dr C. L. Oakley and Dr D. J. Trevan for their constant help and advice.

REFERENCES

GLYNN, J. H. . . . . . . 1935. *Arch. Path.*, xx, 896.
McCLUNG, C. E. . . . . . 1937. Handbook of microscopical technique for workers in animal and plant tissues, 2nd ed., *New York* and *London*, p. 152.
SANDIFORD, B. R. . . . . . 1937. This *Journal*, xlv, 467.
TWORT, F. W. . . . . . . 1924. *J. State Med.*, xxxii, 351.

The Journal of

Pathology and Bacteriology

ACCIDENTAL ELECTROCUTION: WITH DIRECT SHOCK TO THE BRAIN ITSELF

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From the Pathological Department of the West End Hospital for Nervous Diseases, London

(Plates XLVII and XLVIII)

In an annotation on electrical injury in *The Lancet* of 30th September 1944, p. 446, the following statement is made. "It is in the central nervous system that severe electric shock makes its most frequent manifestation, though the current is said to pass only rarely through the brain".

In the following case, the area of immediate contact and point of entrance of the 240-volt alternating current from the main was the right temporal (temporo-sphenoidal) pole of the brain itself, through a trephine opening immediately in front of and slightly above the level of the right ear. This opening had been made for a minor operation on the trigeminal nerve, and an electrically-lit retractor had just been inserted when there was a sudden accidental short-circuit in connection with the resistance, the exact nature of which was not ascertained. There appeared to be no fault in the diathermy apparatus, and it was thought that the resistance in the transformer might possibly have been momentarily defective. An assistant nurse standing between the electric apparatus and the patient also received a shock, and, having her hand on the patient, presumably acted as the conductor of the current from patient to earth.

The accident occurred one afternoon at 2.15 p.m.; the patient immediately became unconscious and remained so until 11.30 a.m. next day, when both respiration and heart's action stopped and she died after an interval of 21¼ hours from the time of the accident.

FIG. 1.—Naked-eye photograph of vertical sagittal section through the lesion on the under surface of the right temporal pole—the dark hæmorrhagic and necrotic patches at the centre of the lower margin—showing the radially-striated appearance of the cortex, described in the text as resembling a section through the gills of a mushroom. The white matter, except for the elongated cavities immediately above the hæmorrhagic patches and the numerous small distended veins (puncta cruenta) elsewhere, appears, to the naked eye, comparatively undamaged. ×⅔.

FIG. 2.—A similar slightly enlarged naked-eye photograph showing details of the disruptive lesion in the grey matter around the sulci in the near neighbourhood of the centre of fig. 1. In the area of white matter at the centre of the lower margin of the photograph, towards the lesion at the right temporal pole, there is more marked disruption than elsewhere in the white matter, in most of which it could be made out only with the aid of a strong hand-lens. ×1·5.

ACCIDENTAL ELECTROCUTION
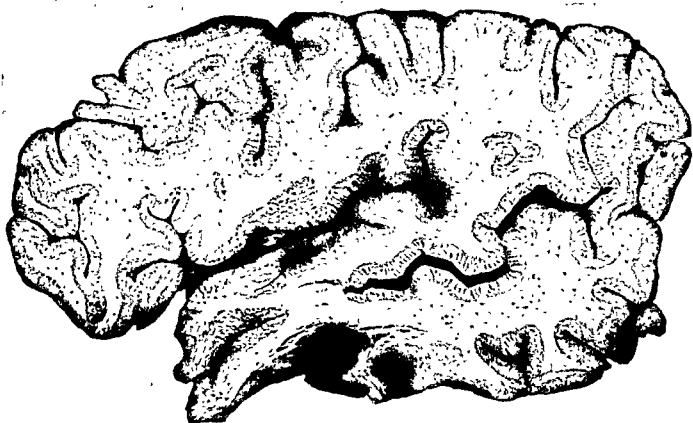
FIG. 1.

FIG. 2.

still attached to the specimen   On the under aspect of the right temporal pole there was a localised necrotic destructive lesion, part of which had been cut away, evidently for microscopical section, but from what remained of the intensely damaged tissues it was obvious that this local lesion had extended superficially over an area about 30 mm in diameter   The outer and posterior part of this damaged area had a dry, eroded, almost worm eaten or finely shattered appearance, likened by some writers to a gunshot wound, and it is reasonable to suppose that this was the site of entry of the powerful electric current which caused the patient's death   This was the only area of the brain to sustain such an intense degree of damage, in the form of an acute necrotic and hæmorrhagic destruction of the cerebral cortex   Around this area of more intense destruction there had spread an extensive and more diffuse finely disruptive type of lesion, most evident to the naked eye in the cortical grey matter and particularly over the outer portion of the right temporal lobe   It had given rise to a curious radially striated appearance (figs 1 and 2), which may be likened to a section through the gills of a mushroom This unusual phenomenon* I attribute to the sudden freeing throughout the moisture containing affected tissues of innumerable minute bubbles of steam or gas—more probably of both—by the combined thermic and electrolytic action of the current   Although to the naked eye this curious change was apparent only in the grey matter of the cortex and basal nuclei, a similar disruptive process but of finer grade had also taken place in the white matter   This, however, except in the immediate neighbourhood of the area of severe destruction, could be made out only with the aid of a strong hand lens   This fine diffuse disruption of brain tissue became progressively less marked towards the middle line, but, on the inner and under aspects of the left temporal pole and as far back as the inner and lower aspects of the left occipital lobe, there was also distinct naked eye evidence of its presence in both grey and white matter   It was not detectable, even with a hand lens, in the cerebellum, pons or medulla   The inner aspect of both frontal lobes and the basal nuclei, especially the right caudate nucleus, also showed a marked degree of the same destructive process

COMMENTARY

In the extensive literature of electrical injuries in man, whether from the action of lighting and industrial currents or of lightning, except for a series of cases described by Professor G B Hassin of Chicago (1933) I have found no recorded examples of lesions in either the grey or white matter of the brain comparable with those in the present case

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* I submitted the photographs of the brain to Professors Percival Bailey and George B Hassin of Chicago for their opinion , both stated that the lesion was, in their experience, unique

The most comprehensive and elaborate work on the subject of electrical injuries is the book *Elektrische Verletzungen* by Stefan Jellinek (1932), professor of electropathology in Vienna, who devoted over thirty years to their study. This author stresses the fact that cerebral and pulmonary œdema are the principal phenomena in fatal cases, and that, in deaths from primary electrical injury to the brain, cerebral œdema, perhaps with a varying amount of hæmorrhage, is the chief and essential post-mortem finding. Other important causes of death are paralysis of the respiratory centres with stoppage of respiration and consequent asphyxia, or in some instances primary cardiac or vasomotor failure. Death may also result from the effects of electrical burns (" elektrischer Verbrennungstod " : Jellinek, *loc. cit.*, pp. 51, 225 and 244) brought about, for example, by the intense heat of the electric arc. Into the controversy as to the propriety of employing the term " electrical burn " in this connection we need not here enter, except to mention that many writers regard it as inappropriate in all connotations. In some respects it is more or less a matter of terminology, and Jellinek himself uses the term in certain connections, though he also stresses the fact that electric trauma is fundamentally *læsio sui generis*, with a pathology of its own : an electric burn is not identical with an ordinary burn. The lesion produced by the electric current he prefers to call electrical necrosis, and it is usually painless and aseptic.

As already stated, I have found nothing in the descriptions of electrical injuries in the writings of Jellinek and others at all comparable with the findings in the present case. All writers who deal with the histopathology of the electrically damaged brain emphasise the degenerative changes produced in the cerebral nerve cells, of all grades and of the usual types—chromatolysis, eccentricity and lysis of the nucleus, etc.—ranging up to complete disintegration and disappearance of many of the cells, while others, even in the same or in neighbouring areas, are apparently unaffected. In the present case, as already explained, only the ordinary routine paraffin sections stained by hæmatoxylin and eosin were available for study, but in these it was noted that many of the cortical nerve-cells, even when situated at the edge of ruptured cavities or dislocated from their previous positions, appeared comparatively unaltered in structure.

The electric current is thought to be conducted most readily along the blood within the vessels, which Jellinek describes as often in a state of spasmodic contraction, with in places disappearance of the lumen and sometimes with patchy destruction of their coats, especially the elastic lamina, resulting in hæmorrhages. Vasomotor paralysis and distension are seen elsewhere, with dilatation of the cerebral veins, which are sometimes thrombosed. The most constant change, however, Jellinek states to be œdema of the brain with, perhaps, varying numbers of petechial and occasionally larger hæmorrhages, and swelling of the endothelial cells of the capillaries, many of which

PLATE XLVIII

J. PATH. BACT.—VOL. LIX

ACCIDENTAL ELECTROCUTION
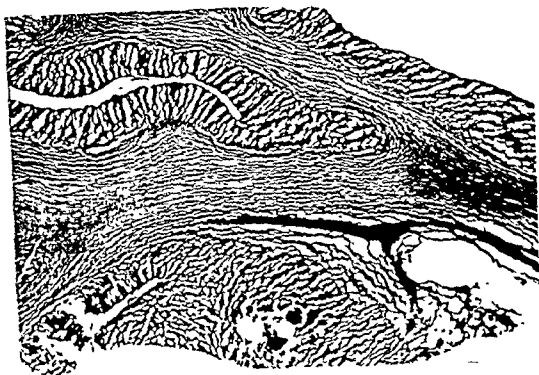
FIG. 3.
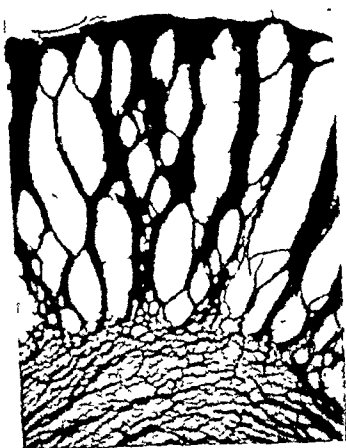
FIG. 4.
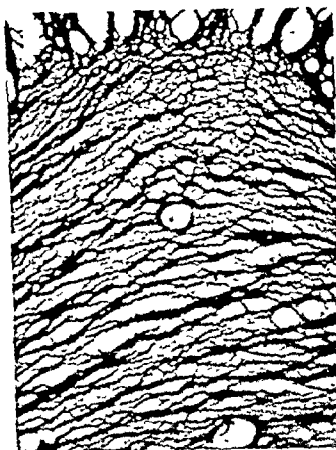
FIG. 5.

are obliterated and destroyed. In the naked-eye slices of this brain, except for the numerous small dilated veins (puncta cruenta) in the white matter (fig. 1), and in the few routine microscopical sections examined, these various changes described by Jellinek were not specially noticeable, though they may well have been obscured by the extensive disruption which had taken place.

As a possible explanation of the relatively more marked destructive changes in the grey as compared with the white matter, it may be suggested that not only is the grey matter softer and more delicate in consistence, but also its liquid content is greater (83-85 per cent. as compared with only some 70 per cent. in the firmer and more fibrillar white matter). There is thus in the grey matter a slightly larger amount of liquid available for conversion into steam or gas on the sudden application of the intense heat and electrolytic action produced by the current.

One of the earliest analogous cases on record is that described by Mott and Schuster (1909-10)—that of a man who lived for seven hours after receiving a powerful electric shock to the back of the head, with extensive external "electric burns" down to the bone, and whose brain showed only some small hæmorrhages in the part of the occipital lobe examined. Microscopically, there was said to be coagulation necrosis, with destruction of nerve-cells and red blood-corpuscles, from the action of the current upon their "osmotic membranes". No disruptive tearing of the cerebral tissues was described, and the small hæmorrhages in the brain and elsewhere were thought to have been due to "the effect of the electric shock on the blood".

Jellinek (loc. cit., p. 231) classifies the forms of death from electric injury as follows:—

(1) *Exitus momentaneus*: immediate death, *e.g.* from lightning.

(2) *Exitus retardatus*: slow death whilst the current is still passing and consciousness is not regained.

(3) *Exitus interruptus*: death where the patient had recovered consciousness, perhaps only for a few seconds, but dies a few minutes later.

(4) *Exitus dilatus*: deferred or delayed death, or, as Jellinek prefers to call it, "late-death" (Spättod), where the victim dies suddenly, perhaps half-an-hour or some hours or even days after the accident.

Jellinek does not apply any scientific name to cases in which, after still further delay, death results from secondary complications, and he notes that, in addition to the various factors connected with the electric current itself, the previous good health or otherwise of the patient may materially influence the period elapsing between the time of the accident and the death of the victim. The present case comes into the 4th of these categories—"*exitus dilatus*"—and to all concerned it was a matter of astonishment that the patient lived as

long as $21\frac{1}{4}$ hours after receiving a full shock direct from the main and applied directly to the brain itself.

Since the publication of Jellinek's book the most authoritative descriptions of the lesions occurring in the brain of electrocuted persons are in the writings of Dr George B. Hassin (1933, 1937), professor of neurology, Chicago. In one of his articles (1933) he describes the changes in the brain in a series of electrocuted criminals examined immediately after practically instantaneous death, a powerful alternating current having passed through the body from forehead to feet in a series of four shocks, the first and third of 2300 volts, each lasting 7 seconds, and second and fourth of 550 volts, duration 52 seconds. "No macroscopic changes, such as manifest hemorrhages or softening, were detected in the meninges, gray or white substance, ventricles, choroid plexus or the basilar blood vessels of the brain".

Microscopically, Dr Hassin describes an "areolar" appearance of the subpial layer, with considerable rarefaction, some tearing of it from the "superimposed pia, and the margins of the separated layers appeared ragged and shaggy. . . . Some areas of the brain tissues were thin, pale and areolar. They often exhibited small rents, tears or cracks, or appeared as narrow slits which were sometimes filled with fragments of brain tissue". In his commentary (*loc. cit.*, p. 1055) Hassin says that: "Of the foregoing changes the most outstanding are the tears of the parenchyma of the brain and of the blood vessel walls"; and on p. 1057 he gives it as his opinion that "the changes observed in legal electrocution are due to purely mechanical factors", similar to those acting in mechanical concussion of the brain or cord, the jarring factor being the electric current, which he does not regard as acting in virtue of either its heat-producing or electrolytic capacity.

In the present case the widespread disruptive lesions in the brain tissue (figs. 3-5) appear to be a greatly exaggerated form of the scattered minute microscopic tears and "fissurations" described by Hassin, but in the present case it is difficult to imagine them produced merely by the mechanical concussing action of the current, rather than by its heat-producing and electrolytic action, as we have suggested above.

SUMMARY

1. The naked-eye and microscopical findings in the brain of an accidentally electrocuted woman aged 45 years are described.

2. A 240-volt alternating current from the main was, owing to a short circuit, applied through an electrically-lit retractor directly to the right temporal lobe, resulting in immediate unconsciousness and death after $21\frac{1}{4}$ hours.

3. The outstanding lesion was a widespread disruptive process in both grey and white matter, demonstrated in the illustrations, and believed to have been produced by the action of the electric current

in liberating bubbles of steam and gas as a result of the intense heat and electrolytic action

4 No description of an extensive cerebral lesion of this nature has been found in the literature of electrical injuries

REFERENCES

HASSIN, G B	1933	*Arch Neurol and Psychiat*, xxx, 1046
,,	1937	*J Nerv and Ment Dis*, lxxxvi, 668
JELLINEK, S	1932	Elektrische Verletzungen, *Leipzig*
MOTT, F W, AND SCHUSTER, E	1909 10	*Proc Roy Soc Med*, iii (Path Sect), 140

ON THE EFFECT OF THE INTERACTION OF STAPHYLOCOCCAL β TOXIN AND GROUP-B STREPTOCOCCAL SUBSTANCE ON RED BLOOD CORPUSCLES AND ITS USE AS A TEST FOR THE IDENTIFICATION OF *STREPTOCOCCUS AGALACTIÆ*

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(PLATES XLIX AND L)

IT is now well established that bovine mastitis is most frequently caused by *Streptococcus agalactiæ*, group B Lancefield. Control measures which aim at eradicating this micro-organism from the cow's udder call for its ready detection in milk samples. Short-cut diagnostic methods have been suggested for this purpose: the Hotis test and direct microscopical examination, for example; but these are presumptive tests and not specific for group-B streptococci.

The more accurate method of plating milk samples in blood agar with or without selective inhibiting substances, followed by serological or biochemical testing of the streptococci, is time-consuming. Any test which will detect *Str. agalactiæ* quickly and accurately on blood-agar plates is therefore useful. The test here described was developed from the observation that all strains of *Str. agalactiæ*, whether hæmolytic or non-hæmolytic, produce an agent which can lyse sheep or ox red cells in the presence of staphylococci producing β toxin (Christie, Atkins and Munch-Petersen, 1944). The toxin itself gives rise to a darkened zone around the colonies on the surface of blood-agar plates, and colonies of *Str. agalactiæ* growing close enough will cause lysis within the darkened zone (fig. 1).

In preliminary tests this phenomenon appeared only with 64 strains of *Str. agalactiæ* and not at all with 72 strains of other streptococci. Agreement with these findings has since been recorded in tests with 200 strains of group B and 395 strains of other streptococci (Munch-Petersen, Christie and Simmons, 1945).

It was also observed that when blood-agar plates with colonies of *Str. agalactiæ* were flooded with cell-free staphylococcal β toxin extensive zones of hæmolysis developed within two hours at 37° C. This observation was the basis of the tests to be described.

MATERIALS AND METHODS

The source of micro-organisms

A total of 650 milk samples was obtained aseptically from individual quarters of cows, most of which were suffering from acute or subacute mastitis. The bacteriological examination included plating of whole milk and of suitable dilutions in blood agar; usually 0·5 ml. of milk was added direct to a tube with 12 ml. of melted agar containing 5 per cent. ox or sheep blood; milk diluted 1 : 10, 1 : 100 or more in saline was also used to ensure a convenient number of isolated colonies and because undiluted milk often causes a confusing darkening of the medium. The plates were incubated at 37° C., and representative colonies were subcultured into serum broth when they had reached a suitable size—usually at 24 or 48 hours; the streptococci thus isolated were tested serologically.

The medium

The basic medium was nutrient agar enriched with sheep or ox blood, but care was taken to use only blood which contained little or no staphylococcal β antitoxin. Tests for suitability were carried out by preparing plates, sowing on the surface a strain of staphylococcus known to produce β toxin, and incubating at 37° C. overnight. The development of a darkened zone around the staphylococcal colonies indicated that the blood was suitable. Such blood could be used equally well whether defibrinated, oxalated or citrated. If much staphylococcal antitoxin was present the red cells were spun down and washed; they were then used in the plates instead of blood.

The staphylococcal β toxin

Staphylococcus S32a (Bryce and Rountree, 1936), which produces β toxin only, was grown in nutrient broth containing 0·1 per cent. agar and 0·1 per cent. glucose and was incubated at 37° C. for three days in an atmosphere of 20 per cent. oxygen and 80 per cent. carbon dioxide. The medium was then clarified by centrifugation, preserved by the addition of 0·02 per cent. merthiolate and stored at 4° C. Toxin stored for five years was as suitable as that freshly prepared; both the toxins used in the tests caused complete lysis of sheep red cells in a dilution of 1 : 1600. The toxin was used in the plate test either undiluted or diluted with saline; a dilution of 1 : 10 gave weak but distinct results, but only very weak reactions were obtained with a dilution of 1 : 15.

The plate test

The plates were marked to show the position of colonies selected for isolation and a record was made of any hæmolysis. One drop of β toxin, usually diluted 1 : 5, was dropped on or near such colonies. The plates were then left at 37° C. and readings were made of induced hæmolysis at intervals of 30 minutes for two hours. Plates were not allowed to cool appreciably during these examinations; otherwise " hot-cold " reactions could lead to error. Lysis during this period within the area covered by the β toxin was taken as proof of the presence of *Str. agalactiæ*. The results were then compared with the microscopical appearance of the organisms on subculture and with their serological reactions.

RESULTS

According to the results of the group precipitin reaction on the streptococci isolated, 365 of the 650 milk samples contained *Str.*

STR AGALACTIÆ
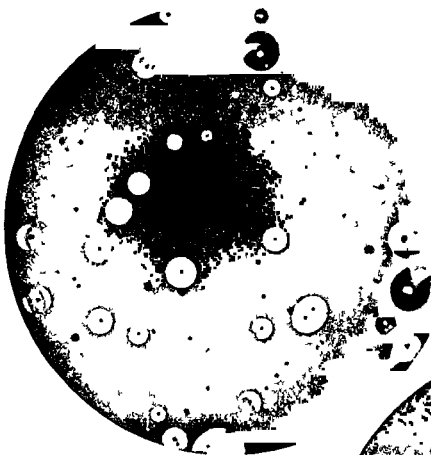
Fig 1.—Blood agar plate containing colonies of β toxin producing staphylococci and non hæmolytic *Str agalactiæ* after incubation for 48 hours at 37° C. The darkened area surrounding the staphylococci have been wholly or partly lysed where *Str agalactiæ* grew sufficiently close. A probable explanation of the crescentic areas of lysis is given in the text. ×1
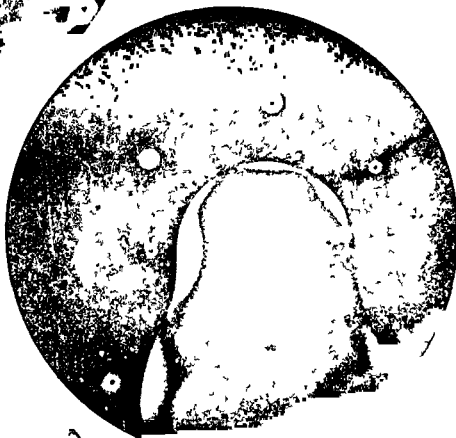
Fig 2.—Crystal violet blood agar plate containing slightly hæmolytic colonies of *Str agalactiæ*, some within and five clearly visible beyond the area covered by the staphylococcal β toxin. The margin of this area is clearly defined, lysis within it has occurred up to 10 mm from each streptococcal colony. The plate was incubated at 37° C for 48 hours and the photograph taken one hour after the application of the toxin. ×1
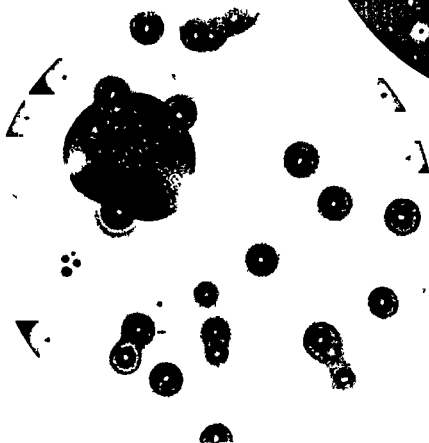
Fig 3.—Blood agar plate with double zone hæmolytic colonies of *Str agalactia*. It was incubated at 37° C for 24 hours, left at room temperature for 6 hours and replaced at 37° C for 24 hours, toxin was then applied so as to touch three colonies and the photograph taken one hour later. ×1

agalactiæ. Staphylococcal β toxin applied to the plates which yielded these streptococci produced faint but distinct hæmolysis around the colonies after 30 minutes at 37° C. The lysis was quite definite after one hour. Figs. 2, 3 and 4 illustrate some of these results.

All strains of *Str. agalactiæ* produced this lysis, whether they were themselves weakly hæmolytic (fig. 2), strongly hæmolytic with double zones (fig. 3) or non-hæmolytic (fig. 4) ; the phenomenon was observed even when the streptococci were growing outside the area covered by the staphylococcal β toxin, if the colonies were within 10 mm. of the edge (figs. 3 and 4).

The addition of crystal violet with either sodium azide or thallium nitrate or acetate in concentrations recommended for rendering blood agar more selective for streptococci did not interfere with the test. The presence of other micro-organisms, none of which gave the reaction, did not prevent or interfere with the lysis produced by *Str. agalactiæ* in the area covered by the staphylococcal β toxin.

Only two samples of milk gave plates with colonies of β toxin-producing staphylococci and *Str. agalactiæ*. Fig. 1 shows one of these. Neither micro-organism produced hæmolysis alone, but where the streptococci were sufficiently close to the darkened zone surrounding the staphylococci definite zones of lysis were produced.

If the streptococcus colony was inside the darkened zone caused by the staphylococcal toxin it was surrounded by a zone of clear hæmolysis ; if it was sufficiently far from the outer edge—more than 10 mm.—no interaction resulted. At intermediate distances clear lysis developed in the intersecting areas of influence, whose shape varied from that of a concavo-convex lens to a segment according to proximity of the colonies. Possible explanations of this are discussed later.

Plates prepared from 285 samples did not contain *Str. agalactiæ*, although there was growth of other streptococci, micrococci, staphylococci and diphtheroid bacilli, either in pure culture or in mixtures. None of these bacteria gave the reaction with staphylococcal β toxin characteristic of group-B streptococci. All these plates were left in the 37° C. incubator overnight after the toxin was applied, and it was then found that two gave a weak reaction. Both contained non-hæmolytic *viridans* streptococci which were not of group B by serological tests. Reactions of this kind (false positives) can be avoided if the final reading is taken not more than two hours after the application of the β toxin. Fig. 5 shows the reaction produced on one of these plates after overnight incubation compared with that on a plate containing a strain of non-hæmolytic *Str. agalactiæ*, one hour after the addition of the toxin.

In a small series of tests run in parallel with those just described, the staphylococcal toxin was incorporated in the medium at the time of plating (one or two drops to 12 ml. of agar). In one such test the plate containing toxin clearly showed the position of the group B

streptococci after overnight incubation at 37° C., although the colonies themselves were hardly visible to the naked eye; the control plate without the toxin, prepared with the same dilution of milk, gave barely visible colonies even after 48 hours at 37° C. Fig. 6 shows these results. However, this method was discontinued, as it was necessary to have control plates without toxin in order to determine the presence of micro-organisms, other than *Str. agalactiæ*, which produce wide zones of normal hæmolysis. Furthermore, some air-borne organisms will give a lytic reaction similar to that produced by *Str. agalactiæ* (Christie and Graydon, 1941), and it might not always be possible to exclude such contaminants from the samples.

DISCUSSION

A study of figs. 1 and 2 would give the impression that the agent produced by *Str. agalactiæ* does not always lyse in the same manner the darkened area caused by the β toxin on blood agar. This apparent variation calls for some comment.

Consider a colony of β toxin-producing staphylococci which is developing on a blood-agar plate within 10 mm. of a colony of *Str. agalactiæ*. The β toxin is revealed by a darkened zone spreading regularly outwards from the colony; the streptococcal agent also spreads regularly but with no obvious alteration to the medium. Where the two zones overlap the lysed area appears. Now, the nearer the two colonies are the wider the lysed area of interception becomes, which might then be expected to take the shape of a true plane lune; in point of fact the area is a concavo-convex lenticule (fig. 2—widest lysed area).

When the streptococcal colony is (say) 9-10 mm. outside the darkened zone the lysed area is almost a segment.

When two streptococcal colonies grow (say) 5 mm. from the β-producing staphylococcus colony and (say) 5-6 mm. from one another, the lysed area may become half-moon-shaped (fig. 1).

Possible explanations of this phenomenon are: (1) that the streptococcal agent is being used up as it advances through the darkened zone, perhaps by being adsorbed; (2) that the red cells (or medium) in the darkened zone resist diffusion, or (3) a combination of both.

When broth containing a 24-hour *Str. agalactiæ* culture is boiled to kill the cells and a drop placed on the darkened zone surrounding a staphylococcal colony so that the centre of the drop is on the edge of this zone, lysis will first appear on the edge, and slightly later extend towards the colony. As incubation proceeds, the darkened zone advances through the remainder of the drop and becomes lysed, but not to the same extent as the edge. Possibly the red cells just undergoing modification by the β toxin are lysed more quickly than those already modified for some time. Why the β toxin diffusing
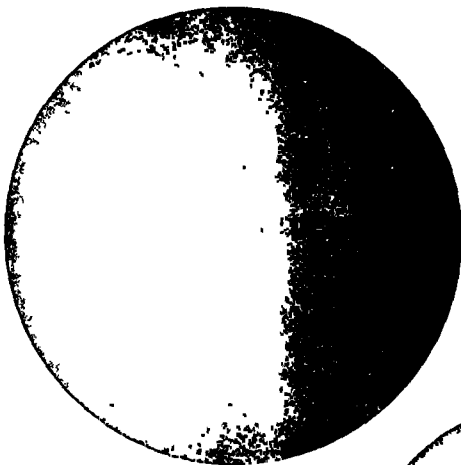
Fig 4 —Blood agar plate with non hæmo lytic colonies of *Str agalactiæ* It wa incubated at 37° C for 36 hours one drop of toxin was added and the photograph taken one hour later   ×1

Fig 5 —Composite figure of two blood agar plates The right half is of a plate contain ing non hæmolytic *viridans* colonies of non group B streptococci which had been incubated at 37° C for 48 hours One half (the upper) was then flooded with staphylo coccal β toxin the plate left at 37° C overnight and the photograph taken Some of the colonies in the area containing toxin simulate slightly hæmolytic *Str agalactiæ* colonies The left half is of a plate contain ing non hæmolytic colonies of group B streptococci which had been incubated at 37° C for 48 hours One half (the upper) was then flooded with toxin and the photograph taken one hour later   ×1
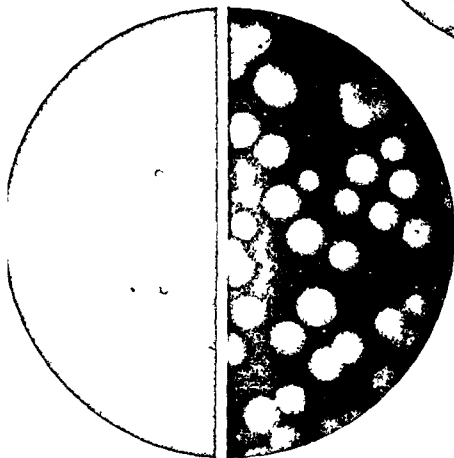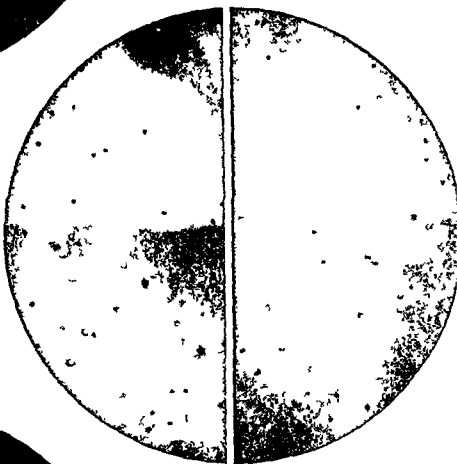
Fig 6 —Composite figure of two blood agar plates the right half is of a plate to which one drop of staphylococcal β toxin had been added at the time of pouring The photo graph was taken after incubation at 37° C overnight The positions of the *Str agalactiæ* colonies are clearly indicated by the presence of the lysed areas, although they are not themselves visible The left half is of a control plate without the toxin incubation was for 48 hours at 37° C but colonies are not yet visible   ×1

through medium with red cells already acted on by the streptococcal agent should not also lyse them completely is not clear. It is paralleled, however, by test-tube experiments with washed red cells, β toxin and streptococcal agent.

In a preliminary series of experiments it was found that the addition to blood agar of cholesterol or washed lysed red cells produced by freezing and thawing did not interfere with or modify the reactions described.

SUMMARY

A test is described for identifying colonies of *Streptococcus agalactiæ* on agar plates containing sheep or ox blood by the addition of staphylococcal β toxin. In less than two hours at 37° C. such colonies will lyse the red cells to a distance of 10 mm. within the area covered by the toxin.

REFERENCES

BRYCE, LUCY M., AND ROUNTREE,   1936.   This *Journal*, xliii, 173.
    PHYLLIS M.
CHRISTIE, R., ATKINS, N. E., AND   1944.   *Austral. J. Exp. Biol. and Med. Sci.*,
    MUNCH-PETERSEN, E.   .   .   .   xxii, 197.
CHRISTIE, R., AND GRAYDON, J. J.   1941.   *Ibid.*, xix, 9.
MUNCH-PETERSEN, E., CHRISTIE,   1945.   *Ibid.*, xxiii, 193.
    R., AND SIMMONS, R. T.

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COAGULASE-POSITIVE STAPHYLOCOCCI RESISTANT TO PENICILLIN

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(Plate LI)

Schnitzer *et al.* (1943) divided penicillin-resistant staphylococci into three types : naturally resistant organisms, those which have acquired resistance through contact with penicillin, and small-colony variants or G forms which have a non-specific resistance to various compounds. Some workers (see Spink *et al*, 1945) further subdivide the second group according to whether the organisms have acquired resistance *in vitro* as a result of serial subculture in penicillin or *in vivo* as a result of penicillin treatment. The present paper is concerned mainly with naturally resistant organisms.

Isolation of resistant strains

Colonies from all plates yielding coagulase positive staphylococci in the routine laboratory during the period of investigation were tested for penicillin sensitivity by the ditch-plate method. The concentration of penicillin in the penicillin agar was 10 units per ml. Nearly always two and sometimes more colonies were picked from each plate and streaked on to a penicillin ditch plate, either directly or after overnight culture in broth. No strain was regarded as resistant unless it grew almost to the junction of the penicillin agar and plain agar or further. In fact minor degrees of resistance were unusual, most organisms appearing to be either frankly resistant or as sensitive as the Oxford staphylococcus, which was included on every plate. Fig. 1 shows a typical result, one strain being frankly resistant. On this plate one strain also appears to be slightly more resistant than the other four, but it was classified as sensitive.

Coagulase positive staphylococci were isolated from 200 patients and sometimes from several specimens from the same patient. Table I summarises the results. In the first series all organisms were also tested for sulphathiazole sensitivity by the method of Harper and Cawston (1945). In all, 25 penicillin-resistant strains were isolated from 200 patients. In 6 cases penicillin resistant and penicillin sensitive organisms were isolated from the same patient and in 4 from the same specimen. In many other cases penicillin resistant staphylococci were isolated from a number of specimens from different sources from the same patient. It will be seen that 5 of the 25 patients with penicillin resistant

staphylococci had been given penicillin before the specimen was taken; only 2 of these had received a full course of treatment.

Incidence of penicillin-resistant Staph. aureus *among 200 patients*

Description of *Staph. aureus* strain	No. of patients		
	Series I	Series II	Total
Coagulase-positive	100	100	200
Penicillin-resistant * . . .	15	10	25
Sulphathiazole-resistant † . .	11	...	...
Penicillin- and sulphathiazole-resistant .	6	...	...

* Three patients of series I and two of series II had been given penicillin before the swab was taken
† Ten patients had been given sulphathiazole before the swab was taken.

Source of penicillin-resistant strains

Table II gives the primary source of isolation of each resistant strain.

TABLE II

Primary source of penicillin-resistant strains

Source	No. of strains
Pyogenic infections . .	5
Conjunctivitis . . .	3
Urine	4
Lactating breasts . .	4
Vagina . . .	4
Nose . . .	3
Skin . . .	1
Air . . .	1
Total . . .	25

Pyogenic infections. Case 1 was one of carcinoma of the larynx. A penicillin-resistant *Staph. aureus* was isolated from an infected tracheotomy wound in association with a Lancefield group-A hæmolytic streptococcus. The sputum contained a penicillin-sensitive staphylococcus, also in association with *Str. pyogenes.* Case 2 was one of bronchiectasis. A penicillin-resistant *Staph. aureus* was isolated in association with a Lancefield group-A hæmolytic strepto-coccus from the sputum in life and from the bronchi and a thrombus of one of the neck veins *post mortem.* This patient had had a full course of penicillin treatment before any specimens were taken. In case 3 there was osteitis following a fractured tibia and fibula. A specimen of pus gave a mixture of penicillin-sensitive and penicillin-resistant *Staph. aureus, Str. pyogenes* and Proteus. The cocci were isolated only after treating a broth emulsion of the original plate with ether and replating (see Pearce, 1946). Case 4 was one of cervical abscess in a child, whose pus gave a pure growth of penicillin-resistant staphylococci. Penicillin treatment (10,000 units 3-hourly) had been started three days before the specimen was taken. Case 5 was one of bilateral otitis media in a child. A penicillin-resistant *Staph. aureus* was isolated from both ears

and from a stomach washout. The ear swabs also grew *Str. pyogenes*. A course of penicillin was started four days before any of the specimens were taken. The *Str. pyogenes* strains from all five cases were penicillin sensitive.

Conjunctivitis. The three cases of conjunctivitis were all in babies and in every case swabs gave a pure growth of a penicillin resistant *Staph. aureus*. One of the three babies had had penicillin eyedrops at birth.

Patients in whom primary isolation was from the urine Two had mixed urinary infections Case 3 was one of nephritis Penicillin resistant *Staph. aureus, Staph. albus, Str. fœcalis* and diphtheroids were isolated from a non-catheter specimen of urine. *Str. pneumoniæ, Str. viridans, H. influenzæ*, neisseriæ and a few colonies of a penicillin resistant *Staph aureus* were isolated from the sputum A nasal swab gave a heavy growth of *Staph aureus*, some of which were penicillin-resistant while others were as sensitive as the Oxford staphylococcus Case 4 was a puerperal patient. A heavy growth of penicillin resistant *Staph. aureus* was obtained from both the urine and a vaginal swab.

Patients in whom primary isolation was from the vagina. Two puerperal cases gave a mixed growth from the vagina, including penicillin resistant *Staph. aureus*. Case 3 was one of toxæmia of pregnancy and nephritis from whom penicillin resistant *Staph. aureus* was isolated from the vagina and penicillin-sensitive *Staph aureus* from the nose. Case 4 was one of vulvo vaginitis in a child. A vaginal swab was overgrown with Proteus, but after ether treatment and re plating penicillin resistant and penicillin sensitive *Staph aureus* and *Str. viridans* were isolated. This child had had two courses of intramuscular penicillin, the first of 4 and the second of 15 days before a swab was taken.

Patients in whom primary isolation was from the lactating breast Case 1 gave a heavy growth of penicillin resistant *Staph aureus* from milk from both breasts. From case 2 penicillin resistant *Staph. aureus* was isolated from both nipples, from a swab taken from an infected Cæsarean wound and from a vaginal swab Case 3 gave a growth of penicillin resistant *Staph. aureus* from a cracked nipple. From case 4 a mixture of penicillin-resistant and penicillin sensitive *Staph. aureus* was isolated from the milk of one breast and penicillin sensitive *Staph. aureus* only from the milk of the other breast.

Nose. Patients in whom primary isolation was from the nose all gave heavy growths of penicillin-resistant *Staph aureus*.

Study of penicillin-resistant strains

All strains found resistant to penicillin on ditch plates were re-tested by the serial-dilution method in broth. The inoculum was 1 drop of a 1:100 dilution of an 18–24-hour broth culture—approximately 50,000 viable organisms. The results with this inoculum are shown in table III

If the inoculum is altered the results are quite different—an observation in striking contrast to the surprisingly constant results irrespective of inoculum size with penicillin-sensitive staphylococci. The effect of inoculum size is shown in table IV. The inocula used were two drops each of 0·02 ml of 18-hour culture, undiluted for the large inoculum and diluted 1 : 10,000 for the small inoculum. The number of bacteria was determined by the rapid viable-count method of Miles and Misra (1938). It will be seen that with the penicillin-sensitive staphylococci—S G. 9727-3 and the Oxford staphylococcus—the results were the same whether the inoculum was many million or less than 1000 bacteria. In contrast to this, with the resistant

strains S.T. 13 and S.G. 9727-1, an inoculum of 13-15 million organisms grew in 200 units per ml. of penicillin, whereas when the inoculum was less than 1000 the maximum concentration of penicillin permitting growth was 0·25 unit per ml. The two strains of *B. subtilis* recom-

TABLE III

Degree of penicillin resistance with an inoculum of 1 drop of a 1 : 100 dilution of an 18-24-hour broth culture (about 50,000 viable organisms)

No. of times more resistant than the Oxford staphylococcus	No. of strains
× 64	1
× 32	6
× 16	10
× 8	7
× 4	1
Total . .	25

TABLE IV

Influence of inoculum size in penicillin-sensitivity tests with penicillin-sensitive and penicillin-resistant organisms

Culture	Maximum concentration of penicillin (u/ml.) permitting growth in 24 hrs.			
	Large inoculum		Small inoculum	
	Inoculum	Penicillin concentration	Inoculum	Penicillin concentration
Staph. ST 13 . . .	13 million	200+	830	0·25
Staph. SG 9727-1 . .	15 ,,	200+	970	0·25
Staph. SG 9727-3 . .	9 ,,	0·03	940	0·03
Oxford Staph. . . .	12·8 ,,	0·03	1280	0·03
B. subtilis 6276 . .	600,000	50	420	No growth in 0·008
B. subtilis 6346 . .	2 million	10	200	0·03

mended by Duthie (1944) for penicillinase production were included in the experiment and showed the same phenomenon as the penicillin-resistant staphylococci. Strain 6276 appeared to be more sensitive to penicillin than the Oxford staphylococcus when an inoculum of 420 organisms was used, whereas an inoculum of 600,000 bacteria grew in 50 units per ml. of penicillin. In another experiment the large inocula were prepared from thrice-washed bacterial cells resuspended in a quantity of broth equal to the original volume. The results were the same.

All the resistant strains and 12 penicillin-sensitive strains of staphylococci were tested for their capacity to destroy penicillin.

The preliminary test to determine simply presence or absence of penicillinase was carried out as follows. Equal quantities of bacterial culture and 4 units per ml. of penicillin were mixed, giving a final concentration of 2 units per ml. of penicillin ; each mixture was then tested for penicillin content by the cylinder-plate method, using the technique described by Hayes (1945). Controls were put up consisting of broth and penicillin only. All 12 sensitive strains mixed with penicillin gave an average diameter of inhibition approximately equal to that of the control. All the 25 resistant strains destroyed the penicillin, so that a circle of inhibition was completely absent. Fig. 2 shows a typical result when 2 resistant and 3 sensitive strains were tested. Fig. 3 shows the comparative results of a resistant strain of *Staph. aureus* (1), *Salm. typhi* (2), *Bact. coli* (3), the Oxford staphylococcus (4), and an uninoculated control (5). The cylinders have been removed in order to show that the two penicillin-destroying cultures and *Salm. typhi* have grown at the bottom of the cylinders. Fig. 4 shows two of the present series of in-vivo penicillin-resistant strains of *Staph. aureus* (1 and 2), an in-vitro acquired resistant culture of the Oxford staphylococcus (3), an ordinary culture of the Oxford staphylococcus (4) and an uninoculated control (5). This demonstrates the difference between in-vitro acquired resistance and natural resistance. All 3 cultures have grown, but the natural resisters have destroyed the penicillin, while the acquired resistant strain has not affected it.

Further tests for penicillinase production were carried out on the in-vivo-resistant organisms. Mixtures were made so that the final concentration of organisms was 5 per cent. and of penicillin 10, 5, 2 and 1 unit per ml. These were tested immediately and after 24 hours' incubation at 37° C. When the tests were put up immediately all the resistant strains had destroyed at least half the penicillin. After 24 hours' incubation so much penicillin had been destroyed that the tubes with 10 units per ml. gave no area of inhibition. In fact most strains were able to destroy considerably more penicillin than this. There was growth at the bottom of the cup with all strains even when the penicillin had not been destroyed. Fig. 5 shows an experiment in which strain S.T. 13 was mixed as above with penicillin of final concentration 20 (1), 10 (2), 5 (3), and 2 (4) units per ml. ; 5 is an uninoculated control of two units per ml. of penicillin. It will be seen that there was growth at the bottom of all the cups (which had been removed) except the control.

All the resistant strains were tested against sulphathiazole by the method of Harper and Cawston and against streptomycin on ditch plates. Seven were so resistant to sulphathiazole that they grew readily in a concentration of 1 : 20,000 or more ; the other 18 were completely inhibited by 1 : 50,000 or less. All showed a sensitivity to streptomycin approximately equal to that of the Oxford staphylococcus.

Fig 1

Fig 2

Fig 3

If penicillin-resistant and penicillin-sensitive strains were isolated from the same patient, cultures were sent to Dr V. D. Allison and Dr Betty Hobbs, who kindly typed them for me. The results are shown in table VI. It will be seen that, except in the case of patient

TABLE VI

Typing of penicillin-resistant and penicillin-sensitive strains of staphylococci from the same patient

Patient no.	Specimen		Colony no.	Reaction to penicillin	Phage reaction	Serological reaction
1	Pus swab	8037	1	Resistant	3A	8
	,, ,,	8038	1	Sensitive	N.T.	III
4	,, ,,	8210	1	Resistant	N.T.	II/4
	,, ,,	8210	2	Sensitive	42C	7211/3537
	,, ,,	8210	3	Resistant	N.T.	II/4
22	Nose swab		1	Sensitive	6/47	III
	,, ,,		2	Resistant	6/47	10667
	Sputum	2721	1	,,	6/47	10667
47	Vaginal swab	9727	1	Resistant	N.T.	3537/B83
	,, ,,	9727	3	Sensitive	N.T.	7211
111	Breast milk	692	1	Resistant	6/47	III
	,, ,,	693	1 .	Sensitive	42E	10153
180	Vaginal swab 9853		1	Resistant	6/47	III
	Throat swab	151	1	Sensitive	N.T.	2411/B83

N.T. = not typable.

22, from whom resistant and sensitive strains showed an identical phage reaction and a closely related serological reaction, the resistant and sensitive strains isolated from the same patient were of different types.

DISCUSSION

Fleming (1942) encountered occasional strains of staphylococci which " were almost insensitive " to penicillin, although he found that the majority were about equally sensitive. Since then a number of authors have isolated penicillin-resistant strains of staphylococci from many sources. Table VII gives the number of penicillin-resistant strains of coagulase-positive staphylococci isolated by various authors before and after treatment with penicillin. Fleming (1942) and Hobby *et al.* (1942) give no details. Rammelkamp and Maxon (1942) used a small inoculum—1000-30,000 organisms—for their tests and thus found that the 2 resistant strains isolated before the onset of treatment were killed by 0·17 and 0·35 units per ml., and the 4 isolated after treatment had begun were killed by from 0·35 to 2·85 units per ml. Kirby (1944) described the production of penicillinase by 7 " naturally " penicillin-resistant strains of *Staph. aureus*. He also tested 7 penicillin-sensitive strains but found they had no penicillinase activity. Anderson *et al.* (1944) studied cultures of *Staph. aureus*

isolated from 32 cases of chronic osteomyelitis. They used the technique described by Rammelkamp and Maxon for penicillin-sensitivity tests, and therefore a small inoculum, so that of the 8

TABLE VII

No. of penicillin-resistant strains of coagulase-positive staphylococci isolated by various authors before and after treatment with penicillin

Author	Date	Coagulase-positive staphylococci resistant to penicillin			
		Before penicillin treatment		After penicillin treatment	
		No. tested	No. resistant	No. tested	No. resistant
Fleming . . .	1942	?	Occasional	...	...
Hobby *et al.* . . .	1942	?	2	...	...
Rammelkamp and Maxon	1942	29	2 slightly	14	4
Kirby	1944	?	7	...	...
Anderson *et al.* . .	1944	32	8 slightly	19	5
Spink *et al.* . . .	1944*a*	68	8	?	1
„ . . .	1945				
Bondi and Dietz . .	1945	66	12	...	...
Gots	1945	?	57	...	...
Gallardo . . .	1945	85	11	?	8
Harley . . .	1946	?	134	?	9·4 per cent.

resistant strains isolated before treatment 7 were inhibited by 0·17 and 1 by 0·35 unit per ml. of penicillin. After treatment 5 more strains were found to be penicillin-resistant and these strains were inhibited only by a concentration of 5·7 units per ml. or more. This was considered to be an acquired penicillin resistance, but it appeared to be unrelated to penicillin dosage. No correlation was noted between results of therapy and the initial variation in penicillin sensitivity, but the resistant strains occurring after treatment had started were regarded as the major cause of penicillin failures. Spink, Ferris and Vivino (1944*a*) studied 68 strains of coagulase-positive staphylococci isolated from patients who had had no penicillin treatment. Their usual inoculum for sensitivity tests was small (100,000-300,000 bacteria) and with this they found that the 8 resistant strains were inhibited by 0·4-0·8 units per ml. They showed, however, that the size of inoculum greatly affected the results and 2 strains tested with a large inoculum grew in 5 units per ml. of penicillin, which was the highest concentration used. They also tested for sulphathiazole sensitivity and found that 19 of the 68 strains were sulphathiazole resistant, but only one of these was also penicillin-resistant. Spink, Hall and Ferris (1945) discussed the clinical significance of natural and acquired penicillin resistance in staphylococci. In spite of having shown that a large inoculum of a naturally resistant strain will grow in at least 5 units per ml. of penicillin, they state that the " sensitivity test as used in this laboratory has failed to reveal a strain of staphylococcus not previously exposed

to penicillin that was not inhibited by 1 unit of penicillin per cubic centimeter of medium, and well over 100 strains have been tested ". On this evidence they conclude that naturally penicillin-resistant strains of *Staph. aureus* are not likely to be a serious cause of failure in penicillin therapy. These authors also contrast cultures which have acquired resistance to penicillin *in vitro* with naturally resistant organisms and those which have developed resistance *in vivo*. Spink, Ferris and Vivino (1944*b*) trained 4 penicillin-sensitive strains of *Staph. aureus* to grow in from 4 to 50 units per ml. These strains with acquired resistance were found to show the morphological and cultural changes described by other workers and were more susceptible to the bactericidal action of human blood. They did not produce penicillinase. In contrast, naturally penicillin-resistant organisms and resistant bacteria occurring *in vivo* after penicillin treatment show none of these changes and all produce penicillinase. Bondi and Dietz (1945) found that 12 of 66 strains of *Staph. aureus* isolated from various sources were penicillin-resistant. They were all penicillinase producers and this was regarded as the cause of their resistance. Gots (1945) studied 57 strains of *Staph. aureus* which needed 1 unit per ml. of penicillin for inhibition and found that all destroyed penicillin, whereas of 42 sensitive strains none produced a penicillin inactivator. Like Bondi and Dietz he regarded the production of penicillinase as the cause of the resistance. Gots also considered that there was a difference between resistance acquired *in vitro* and *in vivo* " inasmuch as we have yet to encounter a resistant strain, isolated directly from an infected process, which is incapable of producing an inactivator ". Gallardo (1945) isolated 85 strains of *Staph. aureus*, of which 11 were penicillin-resistant before treatment; 8 more were found to be sensitive before treatment but resistant after. He gives no details. Harley *et al.* (1946), studying war wounds in India, isolated a large number of penicillin-resistant *Staph. aureus*, and the incidence was much higher among penicillin-treated patients than among those who had not been treated with penicillin. In a group of 43 cases with late infected compound fractures admitted to the base hospital before penicillin treatment in the forward areas, 9·4 per cent. of the *Staph. pyogenes* strains isolated were resistant to penicillin. Of *Staph. pyogenes* strains in wounds with hospital infection 30 per cent. were penicillin-resistant. The degree of penicillin resistance varied enormously, ranging from strains twice as resistant as the Oxford staphylococcus to strains 12,000 times as resistant. One strain was claimed to have increased its resistance more than 40 times in 6 days' treatment. The technique for testing resistance was the serial-dilution method, but the size of inoculum is not stated; perhaps it varied. Any strain was regarded as resistant if it was not inhibited by 0·04 unit per ml. of penicillin.

Conclusions

Three questions are disputed in the literature. First, what is the degree of penicillin resistance of the resistant strains and is it enough to be a serious menace to treatment ? It is clear, I think, from my results that the degree of penicillin resistance recorded in the laboratory depends on the size of the inoculum used for the test of resistance. With the same organism a difference of more than 800-fold was obtained by varying the inoculum. This has also been shown by Luria (1946). As to treatment, it is obvious that a heavy infection with one of these resistant staphylococci would be difficult if not impossible to treat with penicillin.

Second, is the penicillin resistance of these naturally resistant strains solely the result of their capacity to destroy penicillin ? The experiments presented in table IV and fig. 5 make it clear that these organisms have a residual resistance to penicillin apart from their capacity to destroy it.

Third, do staphylococci readily acquire resistance to penicillin *in vivo* during treatment and if so does this type of acquired resistance differ from resistance acquired *in vitro* ? Penicillin-resistant staphylococci are certainly more common in penicillin-treated than in untreated cases, but it does not follow that this results from originally sensitive strains developing resistance. From a number of patients I isolated both penicillin-resistant and penicillin-sensitive strains of *Staph. aureus*, sometimes from the same swab. Therefore, unless many colonies are tested on each occasion, it cannot be accepted that a strain of *Staph. aureus* has developed penicillin resistance during treatment. McKee and Houck (1943) passaged a single strain of *Staph. aureus* through mice treated with penicillin and after 20 such passages no increase in resistance could be demonstrated. Further work along these lines is needed, but this one experiment suggests that staphylococci do not readily acquire resistance to penicillin *in vivo*. In human infection with *Staph. aureus*, the original infection may be due to more than one strain, and reinfection with a further strain is also possible. It is at least possible, therefore, that an increase in penicillin-resistant staphylococci after penicillin treatment may not depend on acquired resistance, but may be the result of a process of selection—naturally sensitive bacteria being quickly destroyed and resistant organisms surviving.

Further evidence in support of this suggestion has become available since the work here described was completed. A patient with carcinoma of the lung had sputum heavily infected with *Staph. aureus*. From a specimen examined soon after admission 31 colonies were tested for their reaction to penicillin by the ditch-plate method. All had a sensitivity approximately equal to that of the Oxford staphylococcus. Twelve days after penicillin treatment (30,000 units 3-hourly) had been begun a second specimen of sputum was in-

vestigated. Cultivation again yielded a heavy growth of *Staph. aureus*; 30 colonies were tested against penicillin and all were found to be highly resistant. Subcultures of 2 colonies from each specimen were sent to Dr V. D. Allison and Dr Betty Hobbs for typing. They found that both cultures from specimen 1 gave a phage reaction 29/52 and were serologically type I, whereas both cultures from specimen 2 gave no phage reaction and were serologically type III/5.

SUMMARY

1. Out of 200 patients yielding cultures of coagulase-positive *Staphylococcus aureus*, penicillin-resistant strains were isolated from 25. In 6 cases the same patient and in 4 cases the same swab yielded both resistant and sensitive strains.

2. The degree of resistance as measured *in vitro* was shown to vary more than 800-fold according to the size of inoculum used.

3. All resistant strains produced a penicillin inactivator.

4. It was impossible to differentiate between resistant and sensitive strains on grounds of morphology, cultural appearances, biochemical reactions or pathogenicity to rabbits.

5. Resistant and sensitive strains isolated from the same patient were usually of different types.

REFERENCES

ANDERSON, D. G., HOWARD, L. G., AND RAMMELKAMP, C. H.   1944.   *Arch. Surg.*, xlix, 245.

BONDI, A., JR., AND DIETZ, C. C. .   1945.   *Proc. Soc. Exp. Biol. and Med.*, lx, 55.

DUTHIE, E. S. .   .   .   .   .   .   1944.   *Brit. J. Exp. Path.*, xxv, 96.

FLEMING, A. .   .   .   .   .   .   1942.   *Lancet*, i, 732.

GALLARDO, E. .   .   .   .   .   .   1945.   *War Med.*, vii, 100.

GOTS, J. S. .   .   .   .   .   .   1945.   *Proc. Soc. Exp. Biol. and Med.*, lx, 165.

HARLEY, H. R. S., BATY, J. A., AND BOWIE, J. H.   1946.   *Brit. Med. J.*, i, 639.

HARPER, G. J., AND CAWSTON, W. C.   1945.   This *Journal*, lvii, 59.

HAYES, W. .   .   .   .   .   .   1945.   This *Journal*, lvii, 457.

HOBBY, GLADYS L., MEYER, K., AND CHAFFEE, ELEANOR   1942.   *Proc. Soc. Exp. Biol. and Med.*, l, 277.

KIRBY, W. M. M. .   .   .   .   .   1944.   *Science*, xcix, 452.

LURIA, S. E. .   .   .   .   .   .   1946.   *Proc. Soc. Exp. Biol. and Med.*, lxi, 46.

MCKEE, CLARA M., AND HOUCK, CAROL L.   1943.   *Ibid.*, liii, 33.

MILES, A. A., AND MISRA, S. S. .   1938.   *J. Hyg.*, Camb., xxxviii, 732.

PEARCE, R. .   .   .   .   .   .   1946.   *Lancet*, i, 347.

RAMMELKAMP, C. H., AND MAXON, THELMA   1942.   *Proc. Soc. Exp. Biol. and Med.*, li, 386.

SCHNITZER, R. J., CAMAGNI, LILIAN J., AND BUCK, MARGARET — 1943. *Proc. Soc. Exp. Biol. and Med.*, liii, 75.

SPINK, W. W., FERRIS, VIDA, AND VIVINO, JEAN J. — 1944a. *Ibid.*, lv, 207.

„ „ „ „ — 1944b. *Ibid.*, lv, 210.

SPINK, W. W., HALL, W. H., AND FERRIS, V. — 1945. *J. Amer. Med. Assoc.*, cxxviii, 555.

INCLUSION BODIES (PROTOZOON-LIKE CELLS) IN THE ORGANS OF INFANTS

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(Plates LII-LIV)

In the examination of the organs of two infants dead from hæmolytic disease of the newborn we observed in many tissues giant cells with cytoplasmic and intranuclear inclusion bodies.

The appearances are identical with those which Ribbert (1904) stated he had first observed in 1881 in the kidneys of a stillborn luetic infant and later in the parotid glands of two non syphilitic infants aged one year and three months respectively. Ribbert's publication was prompted by that of Jesionek and Kiolemenoglou who, earlier in 1904, had described " protozoon cells " in the kidneys, lungs and liver of an eight months syphilitic fœtus   They failed to trace any transition between the large cells and adjacent tissue cells and regarded the whole of the abnormal elements as protozoon parasites. Löwenstein (1907) observed similar bodies in the parotid glands of four out of thirty infants between two and ten months of age   Since then similar bodies have been described in various other sites and in association with diseases other than syphilis by Mouchet (1911) in the bile duct epithelium, by Pettavel (1911) in the thyroid of an infant dying of purpura at ten days of age, and by Smith and Weidman in the kidneys of a stillborn infant (1910-11) and in the lungs of a child dead of pneumonia (1914). In 1922 Jackson recorded " protozoa " in the organs of an infant and emphasised their striking similarity to those she had observed (1920) in the salivary glands of guinea pigs. Up to this time all authors were agreed in regarding the abnormal bodies as some form of protozoan parasite, except Pisanò (1910), who thought they were " embryonic " epithelial cells   Goodpasture and Talbot (1921) observed similar cells in the lungs, kidneys and other organs of a child dying at two months of age with broncho-pneumonia   This infant, the fourth in the family, was markedly anæmic from birth. The mother stated that the second and third children had died at about three months of a similar condition. Goodpasture and Talbot traced the origin of the protozoon like cells to altered tissue cells and drew attention to their marked resemblance to the affected cells in salivary gland disease of guinea pigs (Jackson, 1920), which also they regarded as altered epithelial cells. They recalled that Tyzzer (1905-06) had described intranuclear acidophilic bodies in varicella

Further observations were made on such cells by de Lange (1922) in the renal epithelium of an eight day old child, possibly syphilitic, and by Muller (1922) in three children, one stillborn with " nephritis ", one eight weeks old with hydrocephalus and one two months old, " syphilitic ".

SCHNITZER, R. J., CAMAGNI,   1943.   *Proc. Soc. Exp. Biol. and Med.*, li
   LILIAN J., AND BUCK, MARGARET   75.
SPINK, W. W., FERRIS, VIDA, AND   1944a.   *Ibid.*, lv, 207.
   VIVINO, JEAN J.

    ,,   ,,   ,,   ,,   1944b.   *Ibid.*, lv, 210.
SPINK, W. W., HALL, W. H., AND   1945.   *J. Amer. Med. Assoc.*, cxxviii, 55
   FERRIS, V.

Mrs M.'s serum did not agglutinate the affected child's cells, nor any of the cells in a known panel of all the Rh types. Subsequently the husband's cells were found not to be agglutinated. Mrs M.'s parents were both of group O, the father being homozygous Rh-positive, genotype R_1R_2 (CDe/cDE), while her mother was Rh-negative (cde/cde). In spite of this she had a family of four, of whom Mrs M. was the youngest and without evidence of hæmolytic disease —a finding possibly significant in view of the current belief that the capacity to undergo ready iso-immunisation may itself be inherited (Cappell, 1946 ; Wiener, 1946).

Later, when the delicate Coombs test for the detection of sensitisa-tion of red cells by "incomplete" antibodies became available, it was applied to a variety of cells treated with Mrs M.'s serum, but no evidence of sensitisation was detected, either in a sample of the serum preserved frozen or in fresh samples. There is therefore no evidence that the hæmolytic disease and erythroblastosis present in this case were due to maternal iso-immunisation.

Post-mortem examination

A premature male infant, deeply jaundiced, with very numerous petechiæ in the skin and large ecchymoses over the lumbar region. The heart weighed 15 g. A small amount of deeply jaundiced fluid was present in the pericardial sac. Small petechial hæmorrhages round the A.V. ring. Thymus 4 g. ; a few superficial petechiæ. The peritoneal cavity contained a small quantity of deeply bile-stained fluid which gave a positive direct Hijmans van den Bergh reaction and contained 4 mg. of bilirubin per 100 c.c. Liver 136 g. ; soft and flabby and easily wrinkled ; green necrotic appearance on section ; no atresia of bile ducts. Spleen 52 g. ; much enlarged, dark red and turgid. Kidneys (together 32 g.) bile-stained ; pelves contained some dark brown urine. Suprarenals (together 4 g.). Pancreas normal. The dura mater on the base of the skull was stained bright yellow. No kernicterus ; no cerebral hæmorrhage.

Histology

The *liver* shows severe patchy cytolytic necrosis, with stasis and formation of bile thrombi. Surprisingly little iron is demonstrable. Hæmopoiesis is very abundant in the sinusoids, with large masses of primitive cells and nucleated red cells separating the degenerate liver cells. Large cells with inclusion bodies are present in the epithelium of the small bile-ducts, less conspicuously in the large ducts ; none is observed in the hepatic epithelium. The *spleen* shows marked congestion and extra-medullary hæmopoiesis. The *pancreas* contains an increased proportion of islet tissue and small foci of hæmopoiesis. Numerous cells both in the acini and islets show

intranuclear inclusion bodies and more rarely also cytoplasmic inclusions. In the *lungs* numerous subpleural, interstitial and intra-alveolar hæmorrhages are present, there is hæmopoiesis in the interstitial tissue and solitary large cells with inclusion bodies are seen attached to the alveolar walls and lying free in the alveoli. Large clusters of staphylococci are present in the bronchioles. The *thymus* shows foci of hæmopoiesis. In the *kidneys* the main site of occurrence of the bodies is the tubular epithelium, especially of the convoluted tubules, the lining cells of which are grossly enlarged. The tubular lumina are also dilated, so that the affected tubules are conspicuous even at low magnifications. The interstitial tissue shows patchy hæmopoiesis and foci of lymphocytic infiltration, especially at the boundary zone. Many of the glomeruli are damaged by sclerosis and periglomerular whorling.

Subsequent history

After all the above investigations had been completed we learned that Mrs M. was again pregnant. Her blood was examined and no evidence of iso-immunisation against the husband's cells was found at any time. After a normal gestation and labour the 4th infant was born alive and well; its blood was examined immediately and found to be group O, Rh-positive, genotype R_2r, with no evidence of erythroblastæmia.* This finding supports the hypothesis that the ætiology of the hæmolytic disease in the previous child may have been quite unconnected with maternal iso-immunisation.

Case II

Clinical history

A primipara aet. 22 was delivered of a premature jaundiced infant (38 weeks) who developed generalised purpura and intracranial hæmorrhage from which he died 16 hours later. No investigations were made on the infant during life, and a sample of blood for grouping was not obtained.

Post-mortem examination

This revealed widespread petechial hæmorrhages and massive bleeding into the lungs and intracranially. Microscopic examination showed erythroblastosis of the liver, spleen, lungs and kidneys and intranuclear and cytoplasmic inclusion bodies were found. Unfortunately further details of the findings are not available, but a few sections of the liver, spleen, kidneys and lungs were sent to us for an opinion.

Histology

In general the appearances are strikingly similar to those in our first case. The inclusion bodies are easily identified in the kidneys

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* We are indebted to Dr Ann B. Sandison, director, Blood Transfusion Service, Dundee, for the results of these tests.

and lungs, where they are identical in appearance with those in the previous case but less numerous. In the kidneys they occupy many of the lining cells of the convoluted tubules and cells of Henle's loops; in the lungs they are found exclusively in the lining cells of the pulmonary alveoli and in the liver in the lining cells of the smaller bile ducts. Inclusion bodies were not detected in the spleen.

Blood group investigations

Both parents belong to group O and are Rh-positive, the mother being of genotype Rh_1rh (CDe/cde) and the father of genotype Rh_1Rh_1 (CDe/CDe). The mother's serum does not agglutinate the father's cells at 37° C., at room temperature or at 4° C., nor the cells of any other group-O blood which we have examined. The Rh genotypes are clearly such that no basis for iso-immunisation exists within this system of groups and the complete absence of reaction between the mother's serum and the father's red cells does not suggest that iso-immunisation of the mother had occurred against any other blood-group antigen.

THE MORPHOLOGY OF THE INCLUSION BODIES

Since the abnormal cells are morphologically alike in all situations a detailed description will be given only of the kidneys, where the greater abundance of the changes enables a composite picture to be built up. Here the altered cells lie chiefly in the convoluted tubules, but also, in smaller numbers, in the loops of Henle and in the collecting tubules, where they often appear as single cells amidst an otherwise normal lining. In the narrow loops of Henle the enormously enlarged cells sometimes block the lumen, and in places the normal epithelium is lost so that it is difficult to be sure of the position of the cells and they may appear to lie in the interstitial tissue. Examination of serial sections shows that most if not all of the bodies are actually intratubular. Only two examples have been seen in Malpighian bodies, one in the tuft, the other in the somewhat cubical parietal layer of an incompletely developed glomerulus. The affected tubules are dilated and are easily recognisable under the low power. In some, all the lining cells are affected (figs. 1 and 5); in others, isolated abnormal cells push aside the normal lining elements, which in places then seem to grow under the abnormal elements and to displace them towards the lumen, finally causing their separation into the lumen.

The affected cells are very large—20-35 μ in diameter—and they show a remarkable similarity (figs. 5-8). Transitions between them and the normal cells are only rarely seen, a fact much commented upon by earlier writers and one which materially assisted their former identification as protozoa. We regard as the earliest recognisable stage of development the appearance in cells otherwise normal of a

round, brightly-acidophil intranuclear dot resembling a conspicuous plasmosomic nucleolus (figs. 3 and 6); around this there is a zone of clear nucleoplasm. Cytoplasmic granules are absent from such cells. All stages of transition can be traced between this barely recognisable alteration and the enormously enlarged cells in which the nucleus is replaced by the characteristic "bird's eye" appearance. The more severely affected nuclei are oval, 10-15 μ in greatest diameter and usually eccentric, lying towards the attached border of the cells in situ but more centrally in those which have desquamated. The nuclear membrane of basophilic chromatin shows several condensations or beads, one of which is usually larger than the others; these may conveniently be termed "orbital bodies" (fig. 2). The central acidophil body may be round, oval, reniform or even of horseshoe shape, and the chief orbital body lies opposite the indentation. The central strongly acidophilic inclusion body occupies the greater part of the nucleus and is separated from the nuclear membrane by a zone of clear nucleoplasm crossed in some instances by delicate chromatin threads. The intranuclear bodies are stained black with iron hæma-toxylin, faint blue with alum hæmatoxylin and intense red by the acid fuchsin in Mallory's and Masson's trichromic methods. With eosin-methylene blue and Romanowsky stains they tend to be purplish, but by careful differentiation can be rendered purely eosinophilic; with phloxin-eosin-tartrazine they are stained intensely red. By careful differentiation all these methods reveal the fact that the central body is not homogeneous but is composed of innumerable minute rounded refractile particles closely aggregated into a morula-like mass within which rounded globules like plasmosomic nucleoli can sometimes be seen (figs. 2 and 3). Here and there the nuclear membrane is ruptured and the nucleus appears to be empty; occa-sionally the appearances suggest that the central body is in process of extrusion, but this may be an artefact due to displacement in the cutting of the section. The constituent granules of the intranuclear inclusions are not coloured by Feulgen's reagent. Occasionally two affected nuclei are present in a single cell (fig. 8).

The changes in the cytoplasm of the affected cells are less uniform than the nuclear changes. The cytoplasm is often condensed around the periphery of the cell, giving an appearance like a double-contoured membrane or cuticle (figs. 2 and 3). Many cells show a spongy foamy structure, especially as a crescentic zone between the nucleus and basement membrane, as if due to the presence of finely divided fat. In frozen sections, however, the affected cells show a complete lack of sudanophil material. In occasional cells the supranuclear zone contains one or more large vacuoles, each with a single large acidophilic droplet (fig. 7). The common finding in this site in most of the affected cells is a collection of strongly basophilic granules between nucleus and free border. When numerous, the granules are arranged in regular curved rows and opposite them the double-contoured

INCLUSION BODIES IN RENAL EPITHELIUM OF INFANTS
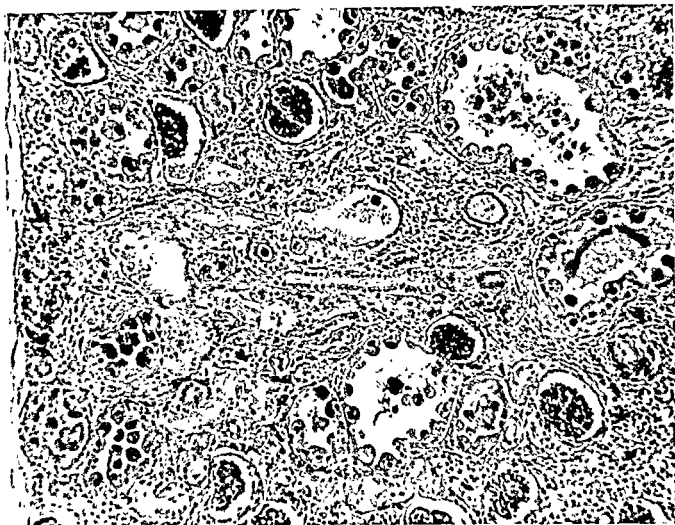
FIG. 1.—Renal cortex showing widespread changes in lining epithelial cells of tubule.
×125.
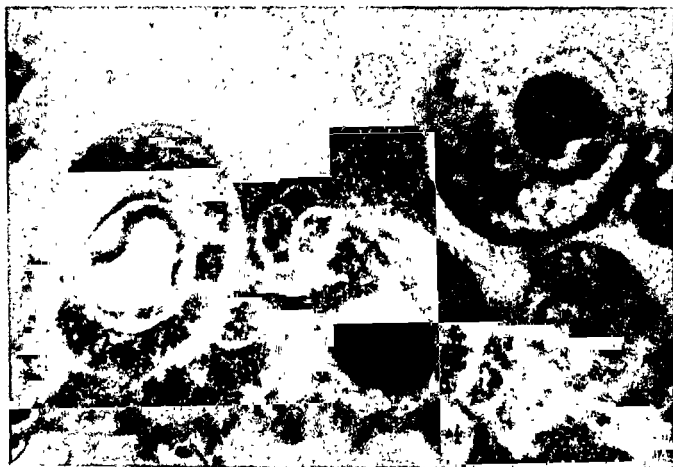
FIG. 2.—Three altered renal tubule epithelial cells: the intranuclear body consists
of a morula of fine closely aggregated granules surrounded by a clear zone of
nucleoplasm. The nuclear membrane shows condensation of the basichromatin
into two bead-like "orbital bodies". The cytoplasm of the basal pole is vacuo-
lated, while the pole towards the lumen contains coarse basophil granules. The
peripheral cytoplasm is condensed into a membrane resembling a cuticle. ×1600.

INCLUSION BODIES IN RENAL EPITHELIUM OF INFANTS

FIG 3 —Renal tubule epithelial cells showing various stages of development of the bodies, up to desquamation and degeneration   Hæmalum and phloxin tartrazine. ×500
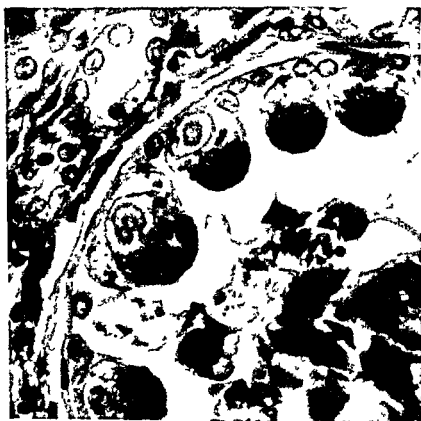
FIG 4 —Basophil cytoplasmic granules arranged in curved rows, the peripheral cuticle is bulging, giving the cells an acorn shape   Dufaycolor photo micrograph.   Gallego Vanucci acetic fuchsin modification of Mallory's stain. ×625

membrane disappears so that this part of the cell bulges through a gap in the " cuticle ", giving the cell an acorn shape (fig. 4). Sometimes at this stage the cytoplasm projects as a clear, lightly stained crescent in front of the regularly arranged rows of granules (fig. 7). The appearances are identical with those described by Farber and Wolbach.

The cytoplasmic granules range in size from minute, just visible dots, to rounded bodies 3-4 μ in diameter. Mostly they are solid, moderately refractile and strongly basophil; sometimes they are surrounded by a clear cytoplasmic zone, as if lying in a fluid-containing vacuole; sometimes the stained substance is in ring form as if deposited upon the inner surface of a vacuole. It is not possible to resolve the larger granules into aggregates of minute bodies with the microscopic equipment available. Sharply differential staining of the granules is achieved by eosin-methylene blue and Romanowsky methods (blue) and with the Gallego-Vanucci acetic-fuchsin modification of Mallory's method, where they are strongly fuchsinophil, in contrast to the intranuclear inclusions, which take up orange G intensely. Prolonged staining with anilin blue will overlay the red colour of the cytoplasmic granules and make them purple or even deep blue (fig. 4). Lendrum's carbacid-fuchsin method (1945) has succeeded in staining them specifically, but the method is capricious on this material. In contrast to the intranuclear bodies the cytoplasmic granules react positively (if weakly) with Feulgen's reagent.

Many of the dilated tubules contain desquamated cells and all stages of degeneration and disintegration are seen (fig. 3). In some tubules, however, the lumen contains innumerable very fine particles which are tinctorially identical with the cytoplasmic granules and which appear to originate in a discharge of granules from the apices of the affected cells.

In other organs the morphology of the affected cells is essentially the same as in the kidney, but as there are fewer cells involved it is less easy to build up a composite picture. Next in frequency of affected cells is the pancreas, where the intranuclear inclusions are identical (fig. 10), though small " early " forms are less easily found. In some of the larger elements the cytoplasm contains ill-defined granules and vacuoles, but only Zenker-formol-fixed material is available, a fixative after which precise staining of the cytoplasmic granules is difficult to obtain. In the lungs both intranuclear and cytoplasmic bodies are present, confined to the cells of the alveolar lining (fig. 9) and wholly absent from the bronchial epithelium, although this site has been much affected in other recorded cases. The inclusions in the bile-duct epithelium present no special features.

Histological examination was also made of the thymus, lymph nodes, spleen, suprarenals, gastro-intestinal tract and heart muscle. In none of these were inclusion bodies found. A notable feature is the failure to detect the characteristic morphological changes in cells other than those which function as epithelium, an observation in

accordance with the findings of Farber and Wolbach. The cells of the reticulo-endothelial system are wholly unaffected. It is therefore worthy of emphasis that in the lungs the affected cells form part of the lining of the air sacs, a point in favour of the existence of a pulmonary alveolar epithelium.

DISCUSSION

There is no doubt that the appearances in the affected cells in these cases are identical with those described and figured by others, and interest centres upon their nature and causation. The view that the characteristic cells are protozoon parasites is now discarded and they must be regarded as tissue cells altered by the effects of some unknown agent. Two points require discussion, (a) the nature of the causal agent, and (b) the circumstances in which it is able to act.

The nature of the intranuclear and cytoplasmic inclusion bodies

Since the work of Goodpasture and Talbot (1921) and Lipschütz (1921) inclusion bodies such as those described in the nuclei in the present cases have been regarded as strongly suggestive of reaction to a virus. The significance of intranuclear inclusions is, however, still unsettled, and Cowdry, Lucas and Fox (1935) emphasise the need for careful scrutiny of the evidence before accepting morphological changes in the nucleus as evidence of the action of a living virus. Intranuclear inclusions have been observed in the tissues of man and animals subjected to the toxic action of heavy metals (Lee, 1933-34; Blackman, 1936; Olitsky and Harford, 1937). Such effects are capricious and the resulting inclusions do not seem to resemble very closely the inclusions in our cases, notably in the absence of the characteristic basophil cytoplasmic granules. Morphologically the nuclear inclusions of our cases correspond very closely with those of type A (Cowdry, 1934) found in herpes, yellow fever, varicella, epizootic abortion in mares (Anderson and Goodpasture, 1942) and in the salivary glands of guinea-pigs and other rodents. These inclusions are acidophilic and contain little or no thymonucleic acid; in thus failing to give the Feulgen reaction the intranuclear bodies in our cases agree with those described by others. As Cowdry *et al.* point out, intranuclear inclusions have been found in the internal organs of animals subjected to a variety of experimental injuries and also in untreated controls (Cowdry and Scott, 1930; Covell, 1932) but these are usually of type B.

Of all the lesions associated with inclusion bodies the only ones which show cytomegaly, intranuclear and cytoplasmic changes identical with those in the organs of infants are the salivary-gland virus diseases of rodents and monkeys, first described in the submaxillary glands of guinea-pigs by Jackson (1920). In several species of animals,
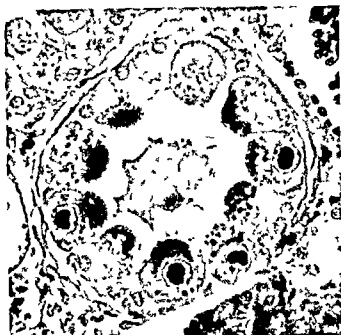
Fig 5 —Intranuclear bodies and cytoplasmic granules with well defined cuticle around some of the cells   × 400
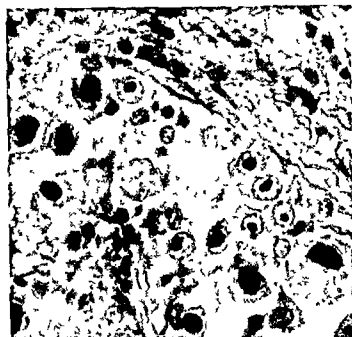
Fig 6 —Renal tubule showing various stages of development of the intranuclear inclusions   × 550.
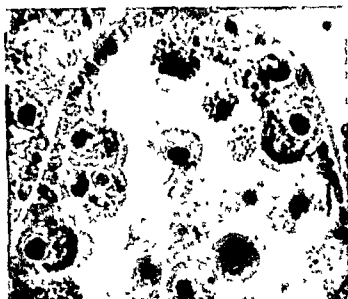
Fig 7 —A collecting tubule containing many desquamated and degenerate cells with numerous affected cells *in situ* Some show the regular curved rows of basophil granules with zone of clear cytoplasm beyond   × 350.
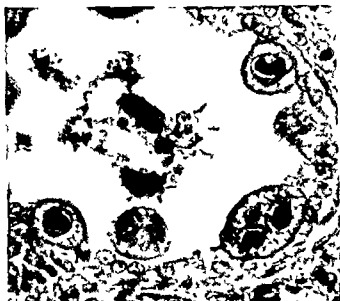
Fig 8 —A binucleated tubule epithelial cell containing two intranuclear inclusions   × 425.
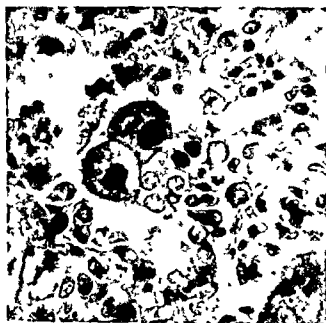
Fig 9 —Two large affected cells in the lining of a pulmonary alveolus   × 450
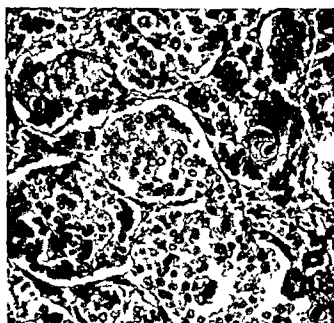
Fig 10 —Pancreas showing three fully developed intranuclear inclusions   × 240.

including *Macacus rhesus*, similar changes may be found in the renal tubular epithelium (Cowdry and Scott, 1935 *a* and *b*). Moore (1944) refers to these as saprophytic viruses and draws attention to the similarity of their associated inclusions to those in the salivary glands and other organs of infants. It is unlikely that the inclusions found in human salivary glands are due to the action of the virus of herpes simplex, because this virus is not reputed to give rise to the characteristic cytoplasmic inclusions which are so conspicuous in affected salivary glands, and all attempts to recover herpes virus from cases with inclusions of salivary-gland type have been unsuccessful. It would, however, be in keeping with the association of salivary-gland inclusions with virus infection in many widely different animal species to infer that the histologically identical lesions in the salivary glands of infants are due to the action of a human strain of salivary gland virus. The fact that under experimental conditions the animal salivary-gland viruses can give rise to generalised lesions, and that such generalisation is more readily induced in the fœtus than in the adult (Markham, 1938) lends further support to the suggestion that the generalised lesions of infants may be due to dissemination of this virus. Since the known salivary-gland viruses are strictly species specific, it is not surprising that attempts have failed to prove by animal inoculation the presence of a virus in human material containing the typical inclusions (Kuttner and Wang, 1934).

Even if it were accepted that the generalised distribution of intranuclear and cytoplasmic inclusions in the organs of infants were due to dissemination of a human strain of salivary-gland virus, there are still many problems unsolved in the relationship between the morphological changes and the intracellular localisation of the virus. In spite of the large amount of work on the salivary gland viruses in recent years, most workers have focussed attention on the intranuclear inclusions ; the cytoplasmic granules have been inadequately described and discussed. They were observed and depicted by the earliest writers but were then regarded as the intracellular organs of the " protozoon parasites ". Nowadays, when the intranuclear salivary inclusions are considered as evidence of the action of a virus on susceptible cells, it may well be asked, are the cytoplasmic bodies to be regarded as another expression of the activity of the same virus or of a second virus ?

Anderson (1942) has shown that simultaneous infection of susceptible cells by two viruses with predominantly cytoplasmic and intranuclear localisation can give rise to two types of inclusion body, each characteristic of the appropriate virus. The histological appearances in the organs of our infants and in the salivary glands of animals would therefore be open to the interpretation that two different agents were acting upon the cells simultaneously. Both in spontaneous and in experimental infections, however, intranuclear inclusions appear earlier than cytoplasmic inclusions (Pearson). The

former may be induced in a variety of cells when infection is introduced by different routes and are abundant in the mononuclear cells of the meningeal exudate produced by intracerebral inoculation of salivary gland material containing inclusions; but cytoplasmic granules are absent. In spite of the presence of abundant intranuclear inclusions in the mononuclears, the meningeal infection is not transmissible by serial passage (Kuttner, 1927). McCordock and Smith (1936) have shown experimentally that intraperitoneal inoculation into a clean strain of mice of broth suspensions or filtrates of mouse salivary glands containing inclusions produced a generalised infection fatal in 4-7 days, with severe lesions in the liver, spleen and pancreas in which intranuclear inclusions were abundant, but basophil cytoplasmic bodies were scanty. It was impossible to propagate the infection by passage of liver and spleen emulsions, whereas from animals surviving 8 days or more the infection could invariably be passed by the injection of emulsions of salivary gland, even when inclusions were scanty. There is thus a striking lack of correlation between the number of intranuclear inclusions and the infectivity of the material, a point also noted by Pearson. There is therefore no decisive proof that the intranuclear inclusions so common in the salivary glands of rodents are specifically related to the virus in the sense that they are actually composed of virus particles, however tempting such an interpretation may be on morphological grounds.

With regard to the cytoplasmic inclusions, there is still less information to enable us to arrive at any proper assessment of their numerical relationship to the infectivity of the material.

There are no corresponding granules in normal cells in these sites. The evidence of morphology seems to us wholly against the interpretation of these cytoplasmic changes as degenerative. The cytoplasmic inclusions of infants and of rodent salivary glands are Gram-negative and are therefore not mere hyaline droplets from protein reabsorption like those often seen in the tubule cells of damaged nephrons. Their intracellular arrangement is highly organised in the form of curved rows of granules which ultimately appear to be discharged into the renal tubular lumen as clouds of minute discrete particles. These appearances are suggestive of the developmental cycle of a living parasite, but our interpretation differs from the conception of earlier writers in regarding the parasite as a virus rather than a protozoon. The relationship between the intranuclear and cytoplasmic bodies is incapable of solution on the material available from these rare human cases.

The association of generalised inclusion bodies with hæmolytic disease

It is noteworthy that 7 out of the 10 examples observed prior to 1930—infants dying in the neonatal period—were classified as

congenital syphilitics, but microscopic or serological confirmation of this was rarely recorded. It seems to us highly significant that the diagnosis " congenital syphilis " has been replaced by " hæmolytic disease of the newborn " as the explanation of the neonatal jaundice in all the examples of generalised visceral inclusion bodies recently published. Since Diamond, Blackfan and Baty (1932) first showed that hydrops fœtalis, icterus gravis neonatorum and congenital anæmia are manifestations of a single pathological process, the clinical and pathological features of this condition have been more. widely recognised, and its essentially hæmolytic origin, first advanced by Parsons, Hawksley and Gittins (1933), has been accepted, as evidenced by the general substitution of the name " hæmolytic disease of the newborn " in place of " erythroblastosis fœtalis ". There were two infants with congenital syphilis in Farber and Wolbach's group with inclusions confined to the salivary glands, but they also recorded the first examples of generalised inclusion bodies in siblings, the younger of whom died of erythroblastosis and the elder of " hæmorrhagic disease of the newborn ". Further cases in hæmolytic disease have been reported by Wanstrom (1933), Andrews and Miller (1935), Vidari (1940) and Kinney (1942).

If we reconsider some of the earlier records in the light of the recent extension of knowledge of the features of hæmolytic disease we might interpret (a) the cellular infiltration of the organs described by Jesionek and Kiolemenoglou as due at least in part to extramedullary hæmopoiesis; (b) the " nephritis " of stillborn infants (Smith and Weidman, 1910-11 ; Müller, 1922) as congenital hydrops ; and (c) the purpura noted by Pettavel and by Farber and Wolbach as the hæmorrhagic manifestations of erythroblastosis.

There is thus a strong possibility that many of the previously described examples of " inclusion bodies " in the newborn occurred in cases of hæmolytic disease. A review of all the older accounts, however, indicates that in some cases the diagnosis of syphilis appears to be justified by the clinical features. The occurrence of the characteristic " protozoon-like bodies " in older children makes it certain that these lesions are not exclusively related to hæmolytic disease, and they have been found in children dying from a wide variety of causes (Farber and Wolbach), including whooping cough (Kinney) and encephalitis (Smith, Lennette and Reames, 1941). This last case is remarkable in that the virus of herpes simplex was recovered from the brain, but in the absence of specific mention of cytoplasmic inclusions it is not certain that this case is of the same nature as our own. Nevertheless the appearances are open to the interpretation that they represent the action of a virus, the tissues of the children being rendered susceptible by debilitating illness of various kinds, and the frequency of hæmolytic disease might reflect merely the importance of this disease as a cause of debility in the newborn.

Hæmolytic disease is now accepted as due chiefly to iso-immunisation of the mother against a blood-group antigen which she herself lacks ; usually the Rh factor is responsible but occasionally the ABO system may be concerned, and, still more rarely, evidence of iso-immunisation is found against some previously unidentified antigen in the red cells of father and infant, when all the commonly recognised blood groups appear compatible (Cappell, 1944, case 10 ; Coombs, Mourant and Race, 1946, case 14 Kells antigen ; Mourant, 1946, Lewis antigen). Callender and Race (1946-47) have also described three new blood-group antigens previously unknown, and clearly the number of these is not exhausted. In the group of cases where paternal and maternal groups are in some respect incompatible but where irregular agglutinins are not demonstrable, the anti-human-globulin test for adsorbed antibody (Coombs, Mourant and Race, 1945) has shown that the infant's cells may be sensitised and the usual ætiology of hæmolytic disease may be presumed. There is still a small group of cases, however, in which the ætiology is obscure, both from failure to demonstrate sensitisation of the infant's cells and from the complete absence of incompatibility of all known groups between the blood of mother and father. In such cases one must enquire whether any other cause for the hæmolysis can be found. As one of us (McFarlane, 1945) has shown, the mere presence of congenital syphilis or other infective condition should not be accepted as the cause of hæmolytic disease unless a concurrent blood group incompatibility and iso-immunisation have been excluded. It is, however, generally agreed that erythroblastosis is a compensatory reaction to persistent hæmolysis, and its presence does not necessarily mean that the hæmolysis was brought about by the action of maternal iso-antibodies.

Our cases are the first examples of hæmolytic disease of the newborn associated with inclusion bodies in which the groups of mother and children have been fully investigated. In them no blood group incompatibility has been detected and attempts to demonstrate an irregular antibody in the maternal sera were unsuccessful, not only against the cells of the affected infant but also against the father's and a large panel of known cells. The indirect Coombs test also failed to reveal any sign of an irregular antibody. We must therefore put these cases into the group having an obscure ætiology, and the question arises whether not only the intracellular inclusions but also the hæmolysis and consequent erythroblastosis may have been the result of a generalised virus infection. If we had been able to show maternal iso-immunisation this would have been an adequate explanation of the evidence of hæmolysis and we might accept the remarkable inclusion bodies as a mere unrelated and incidental finding without causal significance. But we have pointed out, in a critical appraisal of the literature, that in fœtal and neonatal cases they are closely associated with conditions which are now grouped

together as manifestations of hæmolytic disease, namely congenital hydrops and icterus gravis, and it is pertinent to ask whether these inclusions, so typical of virus infections, may be causally related to the hæmolytic fœtal disease by which they are so frequently accompanied. The failure to detect evidence of iso-immunisation and the lack of any recognisable basis for it in both cases increases the probability that the association of the inclusion bodies with hæmolytic disease is more than coincidental.

The successful termination of a further pregnancy in our first case is additional evidence in support of the view that maternal iso-immunisation against a fœtal blood-group antigen was probably not concerned in the causation of the hæmolytic disease in the previous infant.

SUMMARY

1. In the tissues of two infants dead from hæmolytic disease of the newborn widely distributed intranuclear and cytoplasmic inclusion bodies have been found.

2. The affected cells conform to those previously described as "protozoon-like" and the lesions are identical morphologically with those attributed to the action of the salivary-gland virus of rodents and other animals.

3. By analogy with the experimental transmission of the salivary-gland virus in animals, it is suggested that the widespread lesions of infants are probably due to dissemination of a human strain of salivary-gland virus.

4. In view of the common ætiology of hæmolytic disease of the newborn, it is noteworthy that iso-immunisation of the mothers by any of the known blood-group antigens carried by the fœtus has been excluded in both cases.

REFERENCES

ANDERSON, K. . . . . . . 1942. *Amer. J. Path.*, xviii, 577.
ANDERSON, K., AND GOODPASTURE, 1942. *Ibid.*, xviii, 555.
  E. W.
ANDREWS, H. S., AND MILLER, 1935. *Amer. J. Dis. Child.*, l, 673.
  A. J.
BLACKMAN, S. S., JR. . . . . 1936. *Bull. Johns Hopkins Hosp.*, lviii, 384.
CALLENDER, S. T. E., AND RACE, 1946-47. *Ann. Eugenics*, xiii, 102.
  R. R.
CAPPELL, D. F. . . . . . . 1944. *Glasg. Med. J.*, cxlii, 125.
    ,, ... 1946. *Brit. Med. J.*, ii, 601 and 641.
COOMBS, R. R. A., MOURANT, A. 1945. *Brit. J. Exp. Path.*, xxvi, 255.
  E., AND RACE, R. R.
    ,,   ,,   ,,   ,, 1946. *Lancet*, i, 264.

COVELL, W. P. . . . . . . 1932. *Amer. J. Path.*, viii, 151.

COWDRY, E. V. . . . . . . 1934. *Arch. Path.*, xviii, 527.

COWDRY, E. V., LUCAS, A. M., AND 1935. *Amer. J. Path.*, xi, 237.
FOX, H.

COWDRY, E. V., AND SCOTT, G. H. 1930. *Arch. Path.*, ix, 1184.

   ,,   ,,   ,,   ,,   1935a. *Amer. J. Path.*, xi, 647.

   ,,   ,,   ,,   ,,   1935b. *Ibid.*, xi, 659.

DIAMOND, L. K., BLACKFAN, K. D., 1932. *J. Pediatr.*, i, 269.
AND BATY, J. M.

FARBER, S., AND WOLBACH, S. B. 1932. *Amer. J. Path.*, viii, 123.

GOODPASTURE, E. W., AND TAL- 1921. *Amer. J. Dis. Child.*, xxi, 415.
BOT, F. B.

JACKSON, LEILA . . . . 1920. *J. Inf. Dis.*, xxvi, 347.

   ,, ... 1922. *Ibid.*, xxx, 636.

JESIONEK AND KIOLEMENOGLOU . 1904. *Münch. med. Wschr.*, li, 1905.

KINNEY, T. D. . . . . . . 1942. *Amer. J. Path.*, xviii, 799.

KUTTNER, A. G. . . . . . 1927. *J. Exp. Med.*, xlvi, 935.

KUTTNER, A. G., AND WANG, S. H. 1934. *Ibid.*, lx, 773.

DE LANGE, C. . . . . . . 1922. *Arch. path. Anat.*, ccxxxvii, 276.

LEE, J. . . . . 1933-34. *Proc. Soc. Exp. Biol. and Med.*,
xxxi, 383.

LENDRUM, A. C. . . . . 1945. This *Journal*, lvii, 267.

LIPSCHÜTZ, B. . . . . . 1921. *Arch. f. Dermatol. u. Syphilol.*,
cxxxvi, 428.

LÖWENSTEIN, C. . . . . . 1907. *Cbl. allg. Path.*, xviii, 513.

McCORDOCK, H. A., AND SMITH, 1936. *J. Exp. Med.*, lxiii, 303.
M. G.

McFARLANE, M. N. . . . . 1945. *Brit. Med. J.*, ii, 494.

MARKHAM, F. S. . . . . . 1938. *Amer. J. Path.*, xiv, 311.

MOORE, R. A. . . . . . . 1944. A textbook of pathology, *Phila-
delphia* and *London*, p. 470.

MOUCHET, R. . . . . . . 1911. *Arch. méd. exp.*, xxiii, 115.

MOURANT, A. E. . . . . . 1946. *Nature*, clviii, 237.

MÜLLER, J. . . . . 1922. *Arch. path. Anat.*, ccxxxviii, 481.

OLITSKY, P. K., AND HARFORD, 1937. *Amer. J. Path.*, xiii, 729.
C. G.

PARSONS, L. G., HAWKSLEY, J. C., 1933. *Arch. Dis. Childh.*, viii, 159.
AND GITTINS, R.

PEARSON, E. F. . . . . . . 1930. *Amer. J. Path.*, vi, 261.

PETTAVEL, C. A. . . . . . 1911. *Arch. path. Anat.*, ccvi, 1.

PISANÒ, G. . . . . 1910. *Gazz. d. osp.*, xxxi, 249.

RIBBERT, H. . . . . 1904. *Cbl. allg. Path.*, xv, 945.

SMITH, M. G., LENNETTE, E. H., 1941. *Amer. J. Path.*, xvii, 55.
AND REAMES, H. R.

SMITH, A. J., AND WEIDMAN, F. D. 1910-11. *Univ. Penn. Med. Bull.*, xxiii, 255.

   ,,   ,,   ,,   ,, 1914. *Amer. J. Trop. Dis.*, ii, 256.

TYZZER, E. E. . . . . . . 1905-06. *J. Med. Res.*, xiv, 361.

VIDARI, E. . . . . 1940. *Cbl. allg. Path.*, lxxvi, 161.

VONGLAHN, W. C., AND PAPPEN- 1925. *Amer. J. Path.*, i, 445.
HEIMER, A. M.

WAGNER, H. . . . . . . 1930. *Beit. path. Anat.*, lxxxv, 145.

WALZ, K. . . . . 1926. *Verh. dtsch. path. Ges.*, xxi, 236.

WANSTROM, R. C. . . . . 1933. *Amer. J. Path.*, ix, 623.

WIENER, A. S. . . . . . . 1946. *Amer. J. Dis. Child.*, lxxi, 14.

THE PHLOXIN - TARTRAZINE METHOD AS A GENERAL HISTOLOGICAL STAIN AND FOR THE DEMONSTRATION OF INCLUSION BODIES

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(Plate LV)

The original phloxin-tartrazine method was put forward (Lendrum, 1939) as a substitute for the erythrosin-saffron method of Masson, with the claim that it was a more certain way of producing the type of picture given by Masson's beautiful but wayward method. It can give the same colour distribution and the same delicate transparency as Masson's method and although neither is what may be called a primary diagnostic method, both are suitable for the study of cellular (including inflammatory) lesions, for photography and particularly for demonstration. The two purposes of the present contribution are to describe for use in this method a phloxin solution which seems to keep indefinitely in a staining jar, and to detail a particular usage which demonstrates clearly certain inclusion bodies.

The general scheme of the method is nuclear staining by ordinary hæmalum followed by primary cytoplasmic staining with a fluorescein dye ; this in turn is differentiated by treatment with a solution of tartrazine in cellosolve, with resulting removal of the red dye from the collagen and its substitution by the yellow of the tartrazine. It will be noted that ordinary hæmalum is used and Mayer's 1903-04 formula (Lendrum and McFarlane, 1940) is the one preferred ; it is unnecessary to use iron-hæmatoxylin since the section is not going to be exposed to phosphomolybdic or other strong acid. The use of iron-hæmatoxylin, however, is in no way contra-indicated and if it be used as a pure nuclear stain, differentiated for example with the 0.1 to 0.3 per cent. aqueous nitric acid advocated by Cole (1943), the phloxin-tartrazine counterstaining works particularly well ; indeed the use of the nitric acid seems to give an added brilliance to the yellow in collagen. It is to be remembered that this is not a method with the purpose of emphasising the collagen and reticulin, as in the Mallory trichromic method and its endless offspring. The emphasis in the phloxin-tartrazine method is on the elements which retain the

red, namely muscle, blood, fibrin, keratin and various granules; the collagen is not brought into prominence by swelling, as happens with the acid of the usual trichromic methods, and the yellow is of course visually poorer than the red. Unlike the trichromic methods it is best observed in white and not yellow light.

The only red dyes found to be successful in this type of procedure are the fluoresceins and it is considered that the best of these is phloxin (Colour Index no. 778), although even it is improved by the addition of an intensifier to the solution. In my previous paper (1939) two methods of intensifying were given, the addition of either gallic acid or formalin. It was later found, however, that calcium (added to eosin by Conn and Holmes, 1928) is the ideal intensifier for ordinary usage, since the solution keeps so much better, nuclear staining is less affected and differentiation is easier. Of the fluoresceins other than phloxin, erythrosin (C.I. 773) even with calcium chloride is too easily removed from muscle, blood, etc. by the tartrazine, and eosin Y (C.I. 768) is more labile still; rose bengal (C.I. 779) behaves more like phloxin but, although used by some, has not seemed to offer any advantage over it.

The standard method for histological demonstration has now for some four or five years been as follows.

Stain with Mayer's (1903-04) hæmalum for the usual time.
Blue and wash.
Stain with 0·5 per cent. phloxin (C.I. 778) in water containing 0·5 per cent. calcium chloride for 30 minutes in a jar.*
Rinse briefly in water, drain off and replace by a saturated solution of tartrazine NS † in cellosolve (ethylene glycol mono-ethyl ether) from a drop bottle, controlling microscopically.

The tartrazine is very soluble in water and can be easily leached from the section by washing in water; thus one can see, under the low power, if the removal of the red stain has been carried far enough. If the desired degree has been reached a further brief treatment with tartrazine is followed by a rinse with 60 per cent. spirit, 95 per cent. spirit and so to absolute alcohol and xylol. Cellosolve itself is miscible with xylol, alcohol and water, but the tartrazine is insoluble in xylol and only slightly soluble in absolute ethyl alcohol. It is therefore possible with the knowledge of these facts to obtain any desired degree of yellow staining in the final preparation, along with any desired degree of differentiation of the red staining. For some purposes, such as the demonstration of Paneth cells, of phagocytosis of erythrocytes, or of the phloxinophil nucleoli in neoplastic cells, it is worth while

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* This solution keeps for a year and can be reinvigorated by adding a little more calcium chloride. Some phloxins seem to act better if in 70 per cent. alcohol; some are improved by the addition of an equal weight of eosin Y. All react well to calcium but all have the fluoresceins' failing of flocculation.

† Commercial tartrazine is used; the product obtained from Imperial Chemical Industries is cheap and excellent.

prolonging the action of the differentiator and then reducing the final intensity of the yellow staining virtually to nil by thorough washing in water.

The tartrazine cellosolve solution finds frequent other uses in the laboratory : it is our routine to follow Ziehl-Neelsen on sections by staining with hæmalum and then tartrazine, to use very light tartrazine staining after hæmalum and mucicarmine, to follow orcein staining of elastica by hæmalum and then tartrazine, and to follow Perls's iron test with the phloxin-tartrazine method as given above, except that the time in hæmalum is cut to half and treatment with alkali omitted, thus sparing the alkali-soluble prussian blue and leaving the nuclei a reddish colour which contrasts well with the blue. For the above phloxin-tartrazine methods the best fixative is formol-sublimate (one part commercial formalin to nine parts saturated aqueous mercuric chloride) ; prolonged fixation in formalin, Kaiserling, etc. is also suitable, but the bichromate and picric fixatives are not.

By prolonging the action of the tartrazine beyond the stage desirable for ordinary purposes, strikingly different degrees of affinity for phloxin are exhibited by different elements in the section. Thus when the erythrocytes have had their phloxin removed and replaced by tartrazine there is still some phloxin in fibrin, in the granules of the mast cells and eosinophils, in certain granules of glandular epithelium * and in the Russell bodies of the plasma cells ; but when even these have been rendered yellow by continued differentiation (this happens *in the order given*), there remains definite retention of phloxin by the large nucleoli of aberrant neoplastic cells and by certain inclusion bodies. The term phloxinophil inclusion body is used here with a restricted significance to describe a body, intracytoplasmic (figs. 1-3), intranuclear or both, characterised by the extraordinary tenacity with which it retains phloxin. The important technical point is that although the affinity of these structures for phloxin is not absolute and that lengthy treatment with tartrazine will ultimately deprive them of their red colour, there is a significantly wide margin of safety. For example, as one applies the tartrazine to a section of guinea-pig lung and observes the process under the low power of the microscope, the *phloxin is seen to leave the collagen*, then the muscle, then (more slowly) the erythrocytes, and by now, with this power, there seems to be no phloxin retained anywhere in the section. If, however, the section is taken through xylol and mounted, observation with an 8- or 4-mm. objective will show that the granules of the eosinophils have a yellow-orange colour while the Kurloff bodies (Ledingham, 1939) are still strongly phloxin-red (fig. 4). If (and in this lies the technical safety) one wished to decolourise the Kurloff bodies the

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* Moderate phloxinophilia is seen in the granules of the so called "pale" or "pink" epithelium of cystic mastopathy (Lendrum, 1945), a stronger degree is present in the granules of more varied size which appear in the epithelium of localised areas of atrophy of the breast, and in the granules of the Paneth cell (Kerr and Lendrum, 1935-36).

tartrazine differentiation would need to be carried on to something between 18 and 44 hours beyond the stage at which the erythrocytes had been obviously deprived of their red colour. This vivid chromatic isolation of the bodies is in striking contrast to the appearances with say Mann's stain, in which eosinophilic inclusions so closely resemble erythrocytes.

The finding of inclusions and their study in stained sections can explain only a small part of their significance, but accurate knowledge of the frequency with which they occur in relation to recognisable clinical disease will have its evidential value when enough observations have been made. By the use of this simple staining method a number of tissues has now been studied to discover their content of phloxinophil bodies; some of these tissues were from conditions known to be characterised by the presence of inclusion bodies as revealed by other staining methods, and it is perhaps not without practical significance that, in some of these, phloxinophil bodies were found and easily found.

Strongly phloxinophil inclusions of spherical type have been seen in the epithelium of the epididymis of a surgically removed retained testis. These are intranuclear bodies, mainly small and frequently multiple, with occasional clumping to form a morula; a large homogeneous spherical form occurs rarely. These bodies are obviously the same as those figured and described by Heidenhain and Werner (1924, their fig. 9 in colour) as proliferated degenerate nucleolar substance. Scanty spherical inclusions have been seen in the cytoplasm of large cells, probably polyblasts (figs. 1-3), in the intramural inflammatory tissue of tropical · primary phlebitis (Fisher, 1941; Fisher *et al.*, 1947). This type of inclusion resembles closely the form of Russell body which occurs as a single inclusion, showing a smooth, glossy, homogeneous appearance with a surrounding clear halo. In tropical phlebitis, however, the inclusions are contained within cells which are obviously not plasma cells, and they rarely distend the containing cell as all but small Russell bodies commonly do. They appear also to have a rather stronger phloxinophilia. The polymorphous acidophil inclusions of the cytoplasm of the epithelial cells of contagious warts, as studied in three examples of surgically removed keratomas, show a greater phloxinophilia than does the keratin at the surface of the wart (fig. 7). Persistent phloxinophilia has also been demonstrated in the Kurloff bodies (fig. 4) of the guinea-pig (Ledingham), in the intranuclear bodies of the infantile disease recently described by Cappell and McFarlane (1947), in the inclusions of egg membranes (fig. 6) infected with cowpox (Downie and Dumbell, 1947) and in the inclusions (fig. 5) of infantile giant cell pneumonia (Dürck, 1896-97). These last, occurring in histological material from a fatal case of varicella at present under study with Dr Thomas Anderson, had also a fairly strong affinity for two of three samples of ethyl eosin (C.I. 770). Strongly phloxinophil material, homogeneous in structure and rather irregular in shape, has been seen in large cells in the Zenker-fixed

PLATE LV

FIG. 1.—From wall of vein in *tropical phlebitis*: stained hæmalum, phloxin and tartrazine (extended method) and photographed through Ilford red-absorbing filters. Centrally there is a large aberrant cell with a convoluted nucleus (*see* Fisher *et al.*, 1947, p. 412) and immediately below it a cell containing a circular intracytoplasmic inclusion body surrounded by a clear halo. ×1000.

FIG. 2.—Same field photographed through Ilford green-absorbing (magenta) and blue-absorbing (yellow) filters. With this preponderantly red light the red-stained inclusion body is relatively transparent. Comparison with fig. 1 shows that the body is quite opaque there and that it is the only object in the field to show this difference. ×1000.

FIG. 3.—Same field: coloured drawing to show the selective retention of phloxin by the inclusion body. The strong colour contrast, as shown also by the photographs, allows one to find the body at low magnifications. ×1000.

FIG. 4.—Kodachrome of guinea-pig lung: same staining, showing Kurloff bodies; again they are the only structures in the section to retain their phloxin. The dark granules are chlorazol black with which this animal had been injected. ×630.

FIG. 5.—Kodak tricolour transparency of giant-cell pneumonia (case of varicella); same staining, showing phloxinophil material in a multinucleate giant cell in the alveolar wall. These bodies occur in alveolar lining cells even when these cells are small and have only one nucleus. ×160.

FIG. 6.—Kodachrome of egg membrane infected with cowpox: same staining. The large homogeneous inclusions contrast well with the yellowish colour of the nucleated erythrocytes. ×630.

FIG. 7.—Kodachrome of keratinising verruca of face: same staining. The inclusion material has a distinctly stronger phloxinophilia than the superficial keratin. The presence of the phloxinophil material in the cell is not obviously associated with any abnormality of the nucleus that might suggest cellular degeneration.

PLATE LV

PHLOXIN TARTRAZINE

Illustrations of extended method, as for inclusions
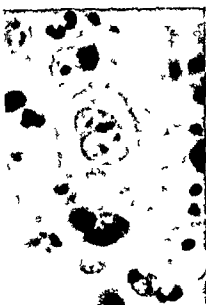
Fig 1

Fig 2

Fig 3
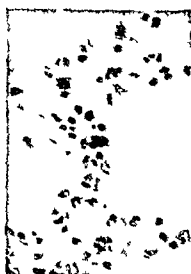
Fig 4
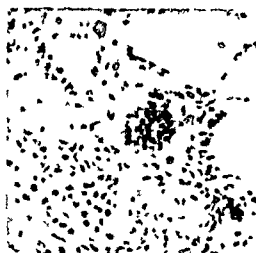
Fig 5

Fig 6
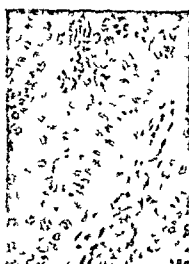
Fig 7

brain of guinea-pigs after passage infection with typhus material (from Dr G. M. Findlay, Dr K. R. Hill and Dr I. Rannie). These inclusions are intracytoplasmic and appear to be related to degeneration of these not clearly identifiable cells.

Not quite all these tissues were fixed in formol-sublimate, but all were subjected to fixatives containing mercuric chloride and were, in general, well fixed; it is possible that some of the negative results described below may have been due to the unsuitability of other methods of fixation. A relatively poor phloxinophilia was seen in the inclusions described by Broadhurst *et al.* (1943) in the buccal epithelium (smears from Dr J. M. Alston; various fixatives), of tissue from ectromelia fixed in Bouin (from Dr R. K. Oag) and of an encephalitic brain fixed in formalin (from Professor Dorothy Russell). Negri bodies in rabid dog brains, after various fixatives including formol-sublimate (from Professor J. W. S. Blacklock and Dr H. E. Hutchison) also showed poor phloxinophilia. Egg membrane infected with variola and fixed in Zenker-formol (from Professor A. W. Downie) failed to show phloxinophilia in the finely granular inclusion material of the ectodermal cells; it is to be remembered that this does not mean that they do not stain with phloxin. Experimental typhus mouse and rabbit lungs after various fixatives including formol-sublimate (from Dr F. Fulton), sewer rat kidneys (Hindle and Coutelen, 1932) and yellow fever monkey material fixed in Bouin or Zenker (both from Professor Hindle), and several granulomata induced in the guinea-pig by the aluminium hydroxide method of Olitsky and Harford (1937) and fixed with formol-sublimate, all failed to show bodies with persistent phloxinophilia.

It is clear, therefore, that the method is not suitable for the demonstration of every type of recognised inclusion body, but in virtue of its technical simplicity and high contrast it does allow the easy and rapid recognition of such inclusion bodies as are strongly phloxinophilic. The appearance presented by the inclusions mentioned above is quite unambiguous and the method would seem to offer . a useful aid both to those whose primary interest is the study of viruses and to the morbid anatomist in his observations on the occurrence of inclusion bodies generally.

Summary

The phloxin-tartrazine method of counterstaining after hæmalum is described as an easily performed and useful procedure, giving the type of result seen after Masson's erythrosin-saffron method.

By prolonged differentiation with the tartrazine there is revealed a strong phloxinophilia of certain inclusion bodies, which can thus be rendered the only red-stained element in the section, enabling them to be picked out with great ease even when scanty.

REFERENCES

BROADHURST, J., MACLEAN, E., AND TAYLOR, I. 1943. *J. Inf. Dis.*, lxxiii, 191.

CAPPELL, D. F., AND MCFARLANE, MARJORY N. 1947. This *Journal*, lix, 385.

COLE, E. C. . . . . 1943. *Stain Technol.*, xviii, 125.

CONN, H. J., AND HOLMES, W. C. 1928. *Ibid.*, iii, 94.

DOWNIE, A. W., AND DUMBELL, K. R. 1947. This *Journal*, lix, 189.

DÜRCK, H. . . . . 1896-97. *Dtsch. Arch. klin. Med.*, lviii, 368.

FISHER, A. C. . . . . . . 1941. *S. Afric. Med. J.*, xv, 131.

FISHER, A. C., FISHER, M. M., AND LENDRUM, A. C. 1947. This *Journal*, lix, 405.

HEIDENHAIN, M., AND WERNER, F. 1924. *Z. ges. Anat.*, I Abt., lxxii, 556.

HINDLE, E., AND COUTELEN, F. . 1932. *C.R. Soc. biol.*, cx, 870.

KERR, A. B., AND LENDRUM, A. C. 1935-36. *Brit. J. Surg.*, xxiii, 615.

LEDINGHAM, J. C. G. . . . . 1939. This *Journal*, xlix, 453.

LENDRUM, A. C. . . . . . 1939. This *Journal*, xlix, 590.

  ,, ... 1945. This *Journal*, lvii, 267.

LENDRUM, A. C., AND MCFARLANE, D. 1940. This *Journal*, l, 381.

OLITSKY, P. K., AND HARFORD, C. G. 1937. *Amer. J. Path.*, xiii, 729.

TROPICAL PRIMARY PHLEBITIS

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(Plates LVI-LVIII)

An acute syndrome of phlebitis and thrombosis of large veins accompanied by pyrexia in previously healthy individuals was first described by one of us (A. C. Fisher) in 1941. The suggestion was then made that this is a clinical entity and confirmation of this view appeared shortly thereafter in Gelfand's book (1944). More recently Manson-Bahr and Charters (1946) have described thrombophlebitis in East African troops, of a type which they state has not previously been recognised; it seems certain enough, however, on the basis of the clinical picture they provide, that they are describing the same disease, thus confirming the view expressed in 1941 that the disease is probably not confined to the copper-mining area of Northern Rhodesia. Since 1941, colleagues in Northern Rhodesia have referred a considerable number of cases to one of us (A. C. F.), and the opportunity has thus been provided to widen our experience of the disease and to obtain material for further histological investigation.

Increasing clinical awareness of this disease emphasises more and more its distinctive characters : (a) it affects young healthy adults leading active lives ; (b) either deep or superficial veins may be involved ; (c) it is acute and usually non-recurrent ; (d) it is accompanied by pyrexia and most of the signs associated with infection and is thus unlikely to be confused with other conditions commonly affecting the systemic veins, such as the thrombophlebitis of Buerger's disease.

There appear to be two main clinical types. The type we have called *phlebitis major* affects the large veins and is accompanied by obvious general upset and pyrexia ; this is the type that most commonly comes under observation. The other, *phlebitis minor*, is essentially a lesion of the smaller veins, obvious enough when it involves the superficial plexus, but thus far only a presumptive diagnosis in the cases where we believe that only deep-seated small veins are involved ; the general upset is much less, thrombosis in the smaller veins naturally causing slighter effects.

<center>CLINICAL ASPECTS</center>

Typical clinical picture of phlebitis major

The patient, a healthy male aged between 20 and 40, is seized with sudden pain over the affected area. In some cases there is a history of malaise during the preceding 24 hours. On examination of, say, the femoral vein (the most commonly affected) there is acute tenderness along its course, associated with spasm of the surrounding thigh muscles. Local œdema and swelling are usually apparent within a few hours of the onset of the pain, and distal venous engorgement appears within the next 24-48 hours in the typical severe case. The height of the pyrexia is variable, in some not rising above 99° F., in others reaching 105°; generally a rather higher temperature occurs with involvement of a large vein. The duration of the fever, from 2 days to 3 weeks, is apparently related to the degree of secondary thrombosis. The œdema and tenderness render examination almost impossible in the early stages, but these symptoms subside within a few days and it is then usually possible to recognise the vein as a smooth, thick, hard cord. In mild cases with minimal evidence of venous obstruction, the thickening and induration of the venous wall are usually gone within 10 days, but in severe cases, with obvious thrombosis, the vein may feel abnormal to the touch for weeks or months and the limb, especially the lower, may be left in a state of permanent œdema. After a less severe attack partial obstruction may be revealed only by the appearance of engorgement and œdema under conditions of stress.

Differential diagnosis

The distinction from tropical myositis is sometimes difficult but usually clear by the second or third day; according to Gelfand, tropical myositis is rarely if ever encountered in the European. The difficulty of distinguishing between phlebitis of the common jugular vein and tropical myositis of the sternomastoid muscle was noted in 1941 (Fisher). Manson-Bahr and Charters report that some 20 of their cases of thrombophlebitis had an acute "stiff-neck", which was neither tropical myositis nor associated with phlebitis of the veins of the neck; in view of the high incidence of involvement of the jugular veins in our series (table), it seems probable that their cases had in fact minor phlebitis in the neck region. In the neck or Scarpa's triangle, acutely inflamed lymph nodes may, during the first day or two, be indistinguishable from tropical phlebitis.

Complications

The only complications observed are those arising from mechanical interference with the circulation, notably where the collateral circulation is less efficient, as in the cerebral and portal veins. Suppuration has not been seen nor any sign of embolism.

Prognosis

A fatal outcome is likely only when the cerebral sinuses or important visceral veins are grossly involved, but since secondary involvement of these may follow onset in a superficial vein of a limb, the prognosis in all cases should be guarded. After a month, however, there seems little likelihood of further thrombosis: the infection has by then run its course. Of the 71 cases recorded, 7 were fatal. As mentioned above, severe thrombotic involvement of a main vein in the leg is usually followed by a fairly massive residual œdema.

TABLE

Phlebitis major: distribution of the lesions

	Left	Right	Total
Superior longitudinal sinus · · · ·	...	...	1
Cavernous sinus · · · · ·	...	...	1
Internal jugular vein · · · ·	2	6	8
External jugular vein · · · ·	1	5	6
Subclavian vein · · · · ·	1	6	7
Axillary vein · · · · ·	6	10	16
Basilic vein · · · · ·	2	1	3
Portal system · · · · ·	...	...	5
Femoral vein · · · · ·	22	12	34
Popliteal vein · · · · ·	5	3	8
Short saphenous vein · · · ·	0	3	3
Total venous lesions · · ·	39	46	92
Number of patients · · · ·			. 68
Patients with multiple lesions . · ·			. 14

Treatment

Sulphonamides were administered in full dosage during the acute febrile stages of the disease without producing any apparent benefit. In the absence of the necessary laboratory controls the use of di-coumarone was avoided and our supply of heparin was insufficient for adequate trial, but since the dangers of the disease are clearly and mainly related to the thrombosis, which we believe to be *secondary* to the lesion of the vessel wall, the use of anticoagulants would seem obviously worth trial.

ILLUSTRATIVE CASES

Cases of phlebitis major

The case numbering is chronological (June 1936 to April 1945). Brief details of all our cases are given in a table which has been deposited with the Librarian, General Library, British Museum (Natural History), London, S.W. 7.

Cerebral involvement. Case 67, male Bantu, age 20–30, miner, was admitted 3.1.44 in a serious condition, unable to give an account of himself. He had a temperature of 103° F. and spastic paralysis of both legs. The blood showed

CLINICAL ASPECTS

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of them recovered without operation and the other revealed at operation what was probably a more severe degree of the same essential lesion.

Case 69.* Male Bantu, age 36, miner, complained of acute pain in the left hypochondrium. There was a history of a recent slight swelling in the left foot and ankle but the details were too vague to justify a retrospective diagnosis of tropical phlebitis. He had a temperature of 103° F. and tenderness with guarding over the splenic area. The signs pointed to subacute rupture of the spleen and operation was performed through a left subcostal incision. The spleen was much enlarged, tense, blue, uniform in appearance and exuding blood over the whole surface. There was much blood in the peritoneal cavity. The spleen was removed and showed no sign of capsular tears, its general appearance suggesting total infarction. The splenic vein contained firm clot but the wall of the vein in the portion accessible to observation appeared normal. He died next day and autopsy was refused.

Case 24, male Bantu, age 16, a schoolboy, complained of severe abdominal pain. He had fever, and guarding over a palpable and tender spleen. The blood smear showed a few malignant tertian rings. A few days later pain and tenderness were also present in the left iliac fossa, then in the thigh and finally in the popliteal space. The veins in the thigh and leg were tender and had the typical rubbery feel. During the following four weeks the fever settled and all local signs gradually disappeared. The contemporaneous existence of a typical phlebitis in the leg and what we may call a splenic syndrome suggests that the latter was really due to mild involvement of the splenic vein.

Cases of phlebitis minor

Case 46, male Bantu, age 20-30, gardener, complained of painful lumps in the arms and legs and a feeling of slight weakness. The nodules, which were somewhat tender, were apparently related to the veins; about half a dozen lay along the line of the superficial veins of the ventral aspect of both forearms, while two or three were in relation to the superficial posterior vein of the calf. In consistence they were firm and rubbery, apparently spherical and about 5 mm. in diameter; they could be moved laterally under the skin but not in the line of the vein. At operation one of these nodules was found to be a localised thickening in the substance of the venous wall; this was removed for microscopy (see p. 412). The remaining nodules gradually disappeared over some months, and neither sequelæ nor recurrences have been noted in the following two years.

Case 48, R.B.E., male European, age 39, laboratory technician, experienced pain at the base of the second right toe, followed by an exquisitely tender swelling. About the fourth day one of the dorsal plexus of veins was palpable as a cord. He got up after 3 days in bed but felt less well, and on the next day pain and swelling reappeared locally. Further rest in bed led to rapid and complete recovery.

Case 49, male Bantu, age 28, houseboy, complained of pain in the left leg, which was slightly œdematous, while there was tenderness of the calf muscles. The temperature, 99° F. on the first day, was normal by the third. Blood culture gave no growth. Within a week he was normal and after further observation he was discharged well on the 20th day. This was regarded as an early stage either of tropical myositis or tropical phlebitis, but the rapid and complete resolution led by exclusion to the presumptive diagnosis of phlebitis minor.

This last case is typical of a disorder commonly seen in Northern Rhodesia which may well be a manifestation of the disease under discussion. Admittedly in none of these is it possible to palpate a thickened tender vein and we wonder if the site of venous involvement may perhaps be small deep-seated vessels,

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* We are indebted to Dr J. H. Dowds for details of this case.

presumed in case 49 to be the venæ comites of the posterior tibial artery. The clinical picture in this group is not unlike that seen in Calabar swelling, but *Filaria loa loa* has not been observed in Northern Rhodesia.

ÆTIOLOGY AND PATHOLOGY

The occurrence of phlebitis in the course of well defined infective diseases such as typhoid, typhus or relapsing fever is well known, and in attempting to show that the present series is a homogeneous group, it is necessary to exclude these obvious predisposing states. In none of the cases was there clinical evidence to suggest the presence of such infections and the laboratory findings provided no unexpected contradiction.   Other obvious predisposing conditions which have to be considered in Africa are helminthic infestation and drepanocytosis. Hæmatological investigations showed an absence of eosinophilia, and of seven cases specially examined for sickling, only two showed this trait.   It is not certain how frequently the sickle-cell condition is complicated by random thrombosis, but the proportion with the trait among the few cases examined is not very different from the 17 per cent. incidence shown by English (1945) to exist in the normal Bantu population.   In general there was only a slight leucocytosis with, in some, a moderate relative lymphocytosis (Fisher); similar findings are given by Manson-Bahr and Charters.   Smears from 41 of the cases revealed malaria (M.T. rings) in only two (nos. 24 and 53).

Since there was no obvious indication of a primary disease on clinical grounds, various laboratory investigations were undertaken in the hope that some positive evidence might be forthcoming. Culture of the blood was carried out on 12 cases, but the only positive finding was a *Streptococcus viridans* from case 42, a finding that we would hesitate to consider as significant.   Of the three Kahn tests recorded, one was strongly positive (38), the others negative (16, 41); two Widal tests were negative (57 and 66), as was the only Weil-Felix test (37). It then remained to investigate the lesion itself, with the idea in mind that the causative factor might be present there and that there might be a characteristic morphological change in the vessel wall.   Our conception of the lesion (during the early part of the study, based largely on the clinical findings) was of damage to the lining of the vein extending over a considerable stretch of the vessel. The findings at necropsy in case 66, however, suggested that the lesion might be intramural, and of severe degree in only a very short segment or segments, despite the much greater length of the vein that appeared clinically to be involved.   Unfortunately the tissues from this case were too autolysed for microscopy, but the appearances in case 70 at exploratory operation seemed to confirm the idea that only a short length of wall was seriously damaged, and with this the microscopical findings agree.
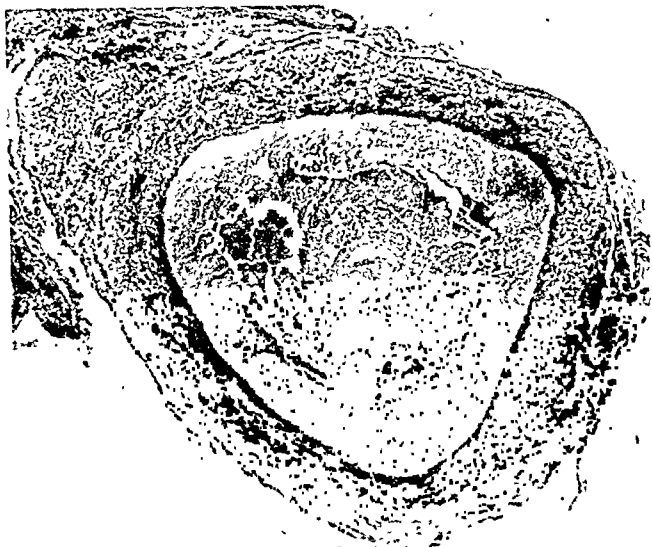
Fig. 1.—Thrombophlebitis of external jugular vein, case 70. The vessel is filled with thrombus, and even at this magnification it is possible to see the disruption of the normal structures of the wall throughout its whole extent shown. ×13.

Fig. 2.—Higher magnification of same vein showing the interruption of the normal (darkly stained) circular structures of the wall, and the intrusion from the periphery of a highly vascularised new tissue. The completeness of apposition of the thrombus to the wall is also noteworthy. ×30.

TROPICAL PHLEBITIS
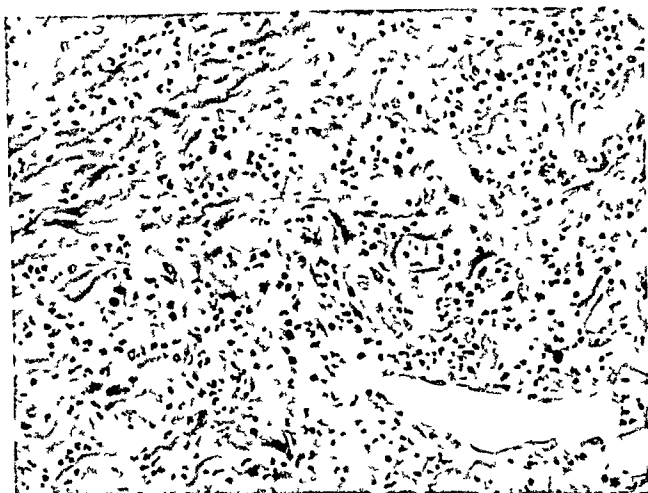
FIG 3—Higher magnification of same vein showing the completeness of the dis
organisation of the wall The thrombus filled lumen is visible in the top left hand
corner The wall is irregularly traversed by young capillaries and in the œdematous
tissue there are many polymorphs   ×250
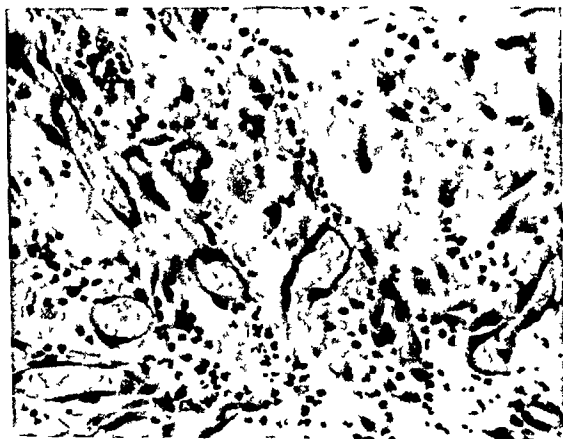
FIG 4—Same vein showing a zone of intramural inflammatory tissue with the very
prominent capillarisation which is so striking a feature of this lesion   ×320

Bacteriological findings

Cultures from the clot obtained at operation on case 70, in which we feel that the primary lesion was accurately identified, yielded no growth (p. 408). Material from the earlier cases may well have been taken from clot which was in fact at some distance from the site of main intramural damage, and in all three cases examined (two by aspiration and one at necropsy) all cultures were sterile.

Histological changes

The findings in case 70 (portion of jugular vein) were not only extraordinary, but have thrown light on the previous biopsy material and permit an interpretation of these earlier specimens which could scarcely have been possible without the help gained from its study. The lesion in case 70, which may reasonably be accepted as the active or acute phase of the disease, is essentially a replacement of the media and intima by actively proliferative vascular tissue with associated polymorph infiltration and the presence of scanty but unequivocal cytoplasmic inclusion bodies.

The thrombus in the lumen is attached to the wall (fig. 1); it is recently formed, shows no organisation and presents no unusual features. As regards the wall of the vein, low-power magnification reveals a remarkable disruption of the normal tissues of the media and intima (fig. 2), the distinction between which is quite lost; the formed collagen sheets are not only separated concentrically from each other by the new œdematous tissue to be described, but they appear to be fractured and the fragments dislocated. The interrupting new tissue seems to take origin from the adventitial zone and to invade the media, where it displays more fully its striking and unusual characters. Under higher magnification (fig. 3) it is seen to consist of new-formed capillaries set in a loose œdematous groundwork in which lie large cells of uncertain nature, and a moderate number of neutrophils. These polymorphs are themselves peculiar in that they show surprisingly good preservation of their structure (figs. 5 and 6); this is obvious in all of them and differs markedly from the state of affairs in the usual pyogenic lesion, where varying degrees of degeneration of the polymorphs can almost invariably be found with ease. The endothelial cells, which by themselves constitute the lining and whole thickness of the walls of these primitive capillaries (fig. 4), are distinctly larger than those in ordinary granulation tissue, even if œdematous, and larger even than those in an actively neoplastic capillary angioma. The appearance of these endothelial cells is quite unusual and their large vesicular nuclei and mildly basophil cytoplasm point to an urgent proliferative activity, as does the finding of numerous mitoses. In the œdematous groundwork between the irregularly disposed capillaries lie large cells of rather similar general appearance to the endothelial cells as

described, with insufficient differentiation to justify the name of fibroblast; some of them show a convoluted vesicular nucleus which recalls the large aberrant cells of Hodgkin's disease, mycosis fungoides and certain reticulin-forming neoplasms of the reticulo-endothelial system. They may in our opinion be accepted as reacting polyblasts, and it is in these that the cytoplasmic inclusion bodies occur.

These bodies are scanty, not more than four or five being present in a transverse section of the whole vein at the level of acute involvement. By the use of the phloxin-tartrazine method as modified to reveal phloxinophil inclusions (Lendrum, 1947) these bodies are stained strongly red; and since the nuclei are blue-grey and all the other tissue elements are stained yellow, the inclusions are easily found even with a relatively low magnification. All are perfectly circular, somewhat hyaline and fairly dense bodies. Some are distinctly smaller than the one illustrated in figs. 1-3 of Lendrum's paper (1947), and only one larger form has been seen; this lies in what appears to be the ghost remnant of a degenerate cell, about the size of the free-lying polyblasts but now without trace of nucleus or other internal structural details.

The sagittal sinus of case 67 was received in diluted glycerin, after fixation in Kaiserling followed by formol-sublimate. It was not possible with the naked eye to determine any focal abnormality in the wall of the vein but, after microscopical examination of many blocks, lesions were found of exactly the same type as those in case 70, although of rather less degree. Scanty cytoplasmic phloxinophil inclusions were also present in this material. The demonstration of similar inclusions was attempted in material from cases 10 and 46 (phlebitis minor). Unfortunately these specimens were fixed in formalin, which rarely proves satisfactory either for the study of cellular detail in a tissue such as a blood vessel or for the successful application of the phloxin-tartrazine staining method. The vein from case 70 was fixed by a method satisfactory for these purposes, namely immersion for 10-14 days in formol-sublimate (Lendrum). The tissue from case 10 would seem, in the light of the findings in case 70 and as was suspected clinically some time after operation, to be a portion of the vein at a little distance away from the site of the actual lesion; the changes in the media (Fisher) can, we consider, be interpreted as a lesser degree of the medial changes now seen in case 70. It is therefore perhaps not surprising that no inclusions were seen in case 10. The lesion in case 46 is basically similar in that there is medial disruption and infiltration by new vessels, but there is in addition more fibrosis, some necrosis of rather indeterminate type, and the presence of small multinucleated cells which might well be a further stage of the cells described above as having convoluted nuclei. No inclusions are seen in these cells, but in some of the smaller wandering cells of the infiltrate there are rather poorly defined, not very strongly phloxinophil areas. The poor fixation of this tissue and

TROPICAL PHLEBITIS
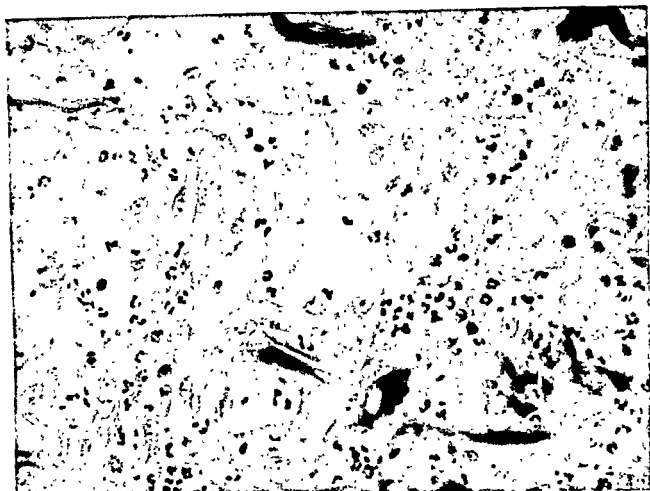
FIG. 5.—Same vein. The darkly-stained masses are remnants of the normal collagen of the vein wall. The surrounding œdematous material is traversed by young capillaries, fibroblasts and other cells with large pale vesicular nuclei. Numerous polymorphs are scattered throughout.   ×470.
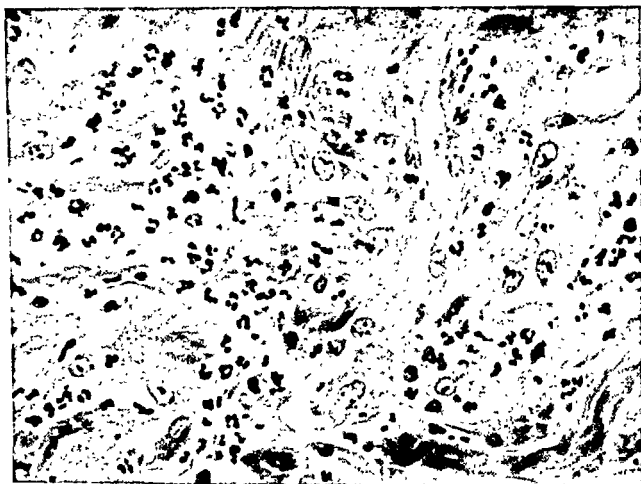
FIG. 6.—Same vein, revealing in greater detail the variation in size and shape of the large vesicular nuclei. Some of the cells are endothelial, others fibroblastic and others again activated cells of the reticulo-endothelial system—reacting polyblasts. The well-preserved appearance of the polymorphs is another feature of the microscopic picture of this disease.   ×520.

the vagueness of the findings make us hesitant, but in view of the decisive appearance in the florid case 70, we are tempted to think that these reddish blobs may be similar in nature.

As a basis for speculation on the sequence of tissue changes in this disease we had material from the florid case 70 removed during the fastigium ; material from case 46, which on clinical grounds belongs to the long duration—low intensity type and which histologically, with its fibrous proliferation, can be accepted as an example of the late stage of the disease ; and finally, but of little value for the present purpose, material from cases 10 and 19 which we presume to have been obtained from the periphery of a lesion. Manson-Bahr and Charters report that they failed to find any evidence of inflammation in the wall of the veins examined microscopically ; it seems not unlikely that they had obtained tissue wide of the essential lesion. From a study of the florid material how far can we peer back into the preceding stages ? Undoubtedly there has been some active stimulus to new capillary formation, a process commonly elicited by the presence of thrombus, fibrin or the gel that overlies the vascularised zone of granulation tissue, but in the vein under study none of these materials is conspicuously present in the wall. Thus further search was made in the sections for anything that could be interpreted as a stimulus to capillary proliferation. Gram's stain and various Romanowsky methods failed to reveal any organisms. The known involvement of endothelial cells by rickettsiæ led to the application of special staining methods for these agents, but again with negative results. It is, of course, not unlikely that methods evolved on Zenker-fixed or properly formalin-fixed material (Nyka, 1944) would prove capricious or useless on our material. It was at this stage that the phloxin-tartrazine method was first used and immediately revealed the inclusion bodies described above. Now since the histological changes in the wall of this vein are quite unlike any ordinary bacterial lesion it is reasonable enough to assume the activity of some ætiological agent other than bacteria, and since this assumption can, in our opinion, be made on the mere morphology of the lesion, the finding of inclusion bodies acquires extra significance and the possibility has to be considered that underlying both the peculiar tissue reaction and the presence of the phloxinophil inclusions is infection with a virus.

The part of the process after the fastigium—the defervescence— can be sufficiently imagined by comparison of cases 46 and 70. Certainly case 46 is justifiably interpreted as the late stage of a lesion with the characters but without the original intensity of the lesion in case 70. This latter lesion, in turn, could well be imagined as proceeding to organisation of the thrombus but accompanied by fibrous contraction in the wall and so to a gross stenosis of the vein with accompanying perivascular fibrosis. Such an idea would fit in well with the impression gained clinically that varying degrees of permanent venous obstruction can be brought about by tropical phlebitis.

FREQUENCY AND DISTRIBUTION

It was obvious from our case reports that the disease occurring among the Bantu is not occupational and the higher incidence in males may well be attributed to the greater number in touch with hospital services (mine workers). This last category is mainly composed of workers, African and European, in the age group 20-40, and it is not surprising that the occurrence in old people has not been observed. One case falls into a younger group (no. 24, age 16), but curiously enough no other cases have been seen in children. Only three definite cases have been encountered in Europeans and the incidence is therefore, if anything, lower than among the Africans. Thus far no seasonal incidence has been established, although we have had a feeling that cases tend to occur in runs. It is therefore particularly interesting that the disease described by Manson-Bahr and Charters, which clinically resembles so closely the disease under discussion, should have been frankly epidemic. Among the African mine workers there was no correlation between length of service and incidence of phlebitis.

The finding of involvement of the splenic vein in this disease raises the question whether some of the cases seen in Northern Rhodesia in the past and diagnosed as splenic softening or splenic abscess of unknown origin, may not have been cases of tropical phlebitis. Further, the observation that cases with a major lesion in the leg are commonly left with a severe postural œdema or a grossly and permanently thickened leg suggested (Fisher) that this form of phlebitis is the underlying cause of " thick leg " or " Serenje leg ", a condition by no means uncommon throughout central Africa.

It would appear probable that tropical phlebitis is not a new disease but merely one which has at last been recognised, since our numbers for each of the years between 1936 and 1945 did not differ greatly ; 1943 with 19 cases gave the highest figure. Then, as this paper was approaching its final draft, Manson-Bahr and Charters published their study of epidemic thrombophlebitis. As we have little doubt that they are describing a manifestation of tropical phlebitis, it seems that the disease can assume an epidemic character, admittedly under the abnormal conditions of military service, and that the geographical distribution of primary tropical phlebitis is now very widespread in Africa. Since the method of spread is still unknown it would be well to consider the possibility that this new thing might come out of Africa. *Ex Africa semper aliquid novi.*

SUMMARY

A study is presented of 71 cases showing an acute phlebitis (tropical primary phlebitis) of unusual clinical type. This has been observed in Africans and Europeans resident in Northern Rhodesia and is believed to be a distinct clinical entity.

An apparently self-limiting acute disease, it may prove fatal if cerebral or visceral veins are affected; in the limbs the obstructive results in the form of severe postural œdema or chronic œdematous thickening of the leg may be surprisingly permanent.

Bacteriological and hæmatological investigations have failed to suggest the cause, but histological examination of the focal lesion of the venous wall has revealed a peculiar type of inflammatory change. In this there is gross interruption of the wall by granulation tissue composed largely of new capillaries, with polymorphs and large polyblasts; in some of the polyblasts it is possible to demonstrate phloxinophil inclusion bodies. The thrombosis would appear to be secondary to the intramural lesion.

REFERENCES

ENGLISH, R. B. . . . . . . 1945. *South African Med. J.*, xix, 431.
FISHER, A. C. . . . . 1941. *Ibid.*, xv, 131.
GELFAND, M. . . . . 1944. The sick African, *Cape Town*.
LENDRUM, A. C. . . . . . 1947. This *Journal*, lix, 399.
MANSON-BAHR, P. E. C., AND 1946. *Lancet*, ii, 333.
  CHARTERS, A. D.
NYKA, W. . . . . 1944. This *Journal*, lvi, 264.

Postscript

M. Gelfand (*Clinical Proceedings*, Cape Town, 1946, v, 381) has described 15 recent cases of idiopathic thrombophlebitis observed in Southern Rhodesia : this disease, the recognition of which he attributes to Fisher, is he believes widespread throughout tropical Africa. He concludes that Manson-Bahr and Charters are describing the same disease. In one of his cases there was a slight pulmonary embolism, in another thrombosis of a radicle of the mesenteric vein (laparotomy), but both recovered. No histological descriptions are given.

FURTHER OBSERVATIONS ON THE CHEMOTHERAPY OF EXPERIMENTAL STAPHYLOCOCCAL INFECTION IN MICE WITH DRUGS OF THE SULPHONAMIDE GROUP, PENICILLIN AND ANTITOXIN

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THE difficulties of observations on experimental staphylococcal infection in mice and assessment of the value of chemotherapeutic agents have been emphasised previously and reference also made to work of others (Browning, 1940).

Properties of staphylococcus strain used

Our method of inoculation was that of Buttle (1937-38), consisting of an intraperitoneal injection of staphylococcus culture in mucin. The strain previously used, after some 250 mouse passages, was subcultured at monthly intervals and occasionally passed through mice in large doses over a period of 3 years. At the end of this time it had lost virulence and efforts to restore it by passage and so forth were unsuccessful. Previously we had found that a useful degree of virulence could not be maintained in frozen or desiccated spleens, or even for a few days in mouse blood-broth cultures kept at −16° C. This is contrary to the experience of Ercoli et al. (1945), who found that blood-agar slants kept in the refrigerator were still virulent after a year. The strain used in the present work was isolated from pyæmia in a lamb ; it had been found to be markedly pathogenic for mice by Mr Angus Foggie, Moredun Institute, who kindly provided it, and this was confirmed in comparison with some 60 other pyogenic strains, mostly of human origin, examined by us. (It may be noted here that in examining strains for virulence in the mouse, 0·5-1 c.c. of an 18 hours' broth culture was injected intraperitoneally, and a most striking feature was the frequency with which such a massive inoculum produced no ill effects whatsoever.) This organism is hæmolytic and coagulase-positive ; it ferments mannitol, liquefies gelatin and forms pigment. To avoid as far as possible the effects of toxin production *in vitro* all inocula were prepared from a suspension of an 18-hour " hormone " agar slope culture in 1 c.c. of 5 per cent. aqueous mucin

(Miller, 1934-35), referred to hereafter as " undiluted suspension ". The organism has been maintained as far as possible by alternate mouse passage and culture of the heart blood on hormone agar. When a mouse failed to die in 24 hours a subculture of an earlier passage was used. Before each experiment, in order to ensure that the organism was in a satisfactory state of virulence for a therapeutic test, four mice were inoculated respectively with 0·5 or 1 c.c. of the undiluted suspension, and 1 : 5, 1 : 50 and 1 : 500 dilutions in mucin and cultures made from the heart blood of those which died within 24 hours. The culture used for the subsequent passage was that which yielded the best growth. Three passages were made in this way. The therapeutic test was not undertaken unless 3 of the 4 mice of the third passage died from the infection within 24 hours. Thus the virulence was controlled almost continuously throughout, and from 83 such tests only 15 cultures were rejected. Table I summarises these virulence

TABLE I

Results of virulence tests in mice

Inoculum	Total no. inoculated	No. dead acutely of septicæmia within			No. of mice with chronic infection		Survivors (slight or no lesions)
		24 hours	1-2 days	3-5 days	Late death with septicæmia	Development of marked local abscess *	
0·5 c.c. undiluted	67	53	4	1	0	5	4
0·5 „ 1 : 5	58	49	1	3	0	3	2
1 „ 1 : 5	9	7	1	0	0	1	0
0·5 „ 1 : 50	60	43	5	0	1	10	1
1 „ 1 : 50	9	7	1	0	0	1	0
0·5 „ 1 : 500	50	17	5	2	2	14	10
1 „ 1 : 500	7	1	0	1	0	4	1

* These animals were chloroformed.

tests. As observed previously, staphylococcal as distinct from streptococcal infections in mice frequently assume a chronic course with the development of local lesions in animals which survive for over 5 days. It is noteworthy that staphylococcal infection in the mouse often presents a clinical picture which resembles human infective diseases, since animals which appear severely ill, and even moribund, may recover from this acute phase. Mice described as having shown chronic infection have either died of staphylococcal septicæmia after upwards of 5 days or appeared well for several days and then developed severe abscesses, usually originating in the needle track of inoculation.

Effect of varying dosage of inoculum. There was a marked falling off in severity of the infection, particularly as regards acute death within 24 hours, when the inoculum was reduced to 1 : 500. Although it was impossible to scrutinise all animals throughout the first 24

hours, it was evident that the undiluted and 1 : 5 inocula tended to kill more quickly than the 1 : 50 dose ; and a considerable proportion of the former were fatal within 8 hours—a period too short to permit of simultaneously administered therapy having any opportunity of success. Consequently it was decided that an inoculum of 0·5 c.c. of a 1 : 20 dilution was optimal for therapeutic trials. This dose contained approximately 10 times the number of organisms previously used.

Treatment with sulphonamide drugs

The drugs of the sulphonamide group used in our earlier experiments were divided broadly into soluble and insoluble compounds, the former represented largely by prontosil and sulphanilamide and the latter, which proved to be the more effective, by sulphapyridine and monoacetyl diamino diphenyl sulphone, these being equally active. In addition, the advantage of combined therapy with staphylococcal antitoxin was demonstrated. Farrell (1940) has also shown the efficacy of antitoxin in his carefully worked-out method for the relative evaluation of sulphonamide drugs in staphylococcal infection in mice. The value of this combined therapy has since been confirmed by Kolmer and Brown (1942), who found sulphapyridine and sulphathiazole especially active in this way. The results of Steinfield *et al.* (1942-43) with sulphathiazole have been similar. Like ourselves, however, they did not confirm the 100 per cent. efficacy of antitoxin along with sulphanilamide originally claimed by De and Basu (1938), who observed their animals only for 4 days. Combined therapy has since been adopted clinically to some extent, though apparently discontinued since penicillin became available.

In the present experiments the general scheme of infection and treatment followed closely that already described. In therapeutic tests infection was by intraperitoneal inoculation of 0·5 c.c. of a 1 : 20 dilution in mucin of the usual suspension of an 18-hour slope culture. Antitoxin, when given, was injected subcutaneously in a dose of 120-150 international units 18 hours before infection. The rationale of this mode of administration has since been confirmed by Farrell and Kitching (1940). The drug was injected subcutaneously as a dispersion, the dose shown being that for a 20-g. mouse. Three doses were administered—immediately after inoculation, and 5 hrs. and 24 hrs. later. All dosage was based on experience of the most effective treatment of highly virulent streptococcal infections in mice (M. W. Leckie, unpublished).

Monoacetyl diamino diphenyl sulphone was included in the present experiment (see table II, which summarises 8 series) in order to establish a comparison with our previous work ; and the newer, more effective compounds, sulphathiazole and sulphamezathine (sulphadimethylpyrimidine, sulphamethazine), were also used. All drugs were administered in combination with antitoxin, and mice treated

with antitoxin alone served as controls. Table II shows the mice grouped as survivors or as having run an acute or chronic course, as in table I. The proportion of survivors treated with antitoxin alone

TABLE II

Results of treatment of staphylococcal infection in mice with sulphonamide drugs and antitoxin

Drug and dosage at 0, 5 and 24 hours after inoculation	No. of mice	No. of acute deaths	Average length of life in days of mice dying within 5 days	No. of mice with chronic infection	Survivors	
					No.	Per cent.
Monoacetyl diamino diphenyl sulphone (6·6 mg., 4 mg. × 2) *	26	20	1·3	1	5	19·2
" " " " " " plus antitoxin	72	40	1·9	6	26	36·1
Sulphathiazole (10 mg., 6·6 mg. × 2)	26	15	1·3	7	4	15·4
" " " " " plus antitoxin	74	22	2·2	7	45	60·8
Sulphamezathine plus antitoxin (10 mg., 6·6 mg. × 2)	36	12	1·4	8	16	44·4
Antitoxin alone . . .	72	36	1·6	10	26	36·1
Untreated controls . . .	74	52	1·1	18	4	5·4

* Dosage :—6·6 mg., 4 mg. × 2 means, for a 20-g. mouse, 6·6 mg. subcutaneously at time of inoculation, followed by 4 mg. 5 and 24 hours later : other doses similarly.
In all animals receiving antitoxin, this was injected subcutaneously 18 hours before inoculation: dose = 120-150 international units for a 20-g. mouse.

was greater than with either sulphone or sulphathiazole alone—36·1 per cent. compared with 19·2 and 15·4 per cent. respectively. The result of combined antitoxin *plus* sulphonamide therapy showed 60·8 per cent. survivors with sulphathiazole, 44·4 per cent. with sulphamezathine and 36·1 per cent. with sulphone. It seems remarkable that the last figure represents a therapeutic result no better than with antitoxin alone (72 animals were employed in each series). Also the result differs from those of all the other experiments in which combined therapy was employed, and these include penicillin, as well as from our previous work. We consider, however, that the combined toxicity of the particular drug product *plus* infection was a limiting factor. In contrast to the finding of Ercoli *et al.* mucin did not increase the toxicity of the drug in the dosage employed. Our mucin suspension was always prepared freshly for each day's experiments, because it tended to become toxic on keeping.

With this strain of staphylococcus (except for four animals treated with antitoxin *plus* sulphathiazole), all the 126 survivors after treatment with sulphonamide or antitoxin alone or combined, as well as the few untreated survivors, developed some local infection of the abdominal wall at the site of inoculation. Healing occurred ultimately, and when examined *post mortem* after 3-4 weeks, all proved to be free from staphylococcal infection as judged both by the naked-eye appearance of organs and negative cultures of heart blood. As

previously noted (Browning, 1940), it is essential to observe for a period of at least three weeks, animals which appear well, and in assessing the results of therapy a considerable number of mice must be used. Recognition of these points appears in the recent general trend of experimental work with staphylococci. Our results with sulpha-thiazole *plus* antitoxin as compared with sulphapyridine *plus* antitoxin confirm the general finding that the former is the more active (Barlow and Homburger, 1939; Bliss and Ott, 1940; Kolmer and Brown, 1942; Ercoli *et al.*, 1945).

Treatment with penicillin

The experiments with penicillin were similar in all respects to the above, treatment being given at the same intervals in the form of an aqueous solution. This plan has since been recommended by Jawetz (1946). Table III (which summarises 8 series) shows that a

TABLE III

Results of treatment of staphylococcal infections in mice with penicillin and antitoxin

Dosage at 0, 5 and 24 hours after inoculation ‡	No. of mice	No. of acute deaths	Average length of life in days, of mice dying within 5 days	No. of mice with chronic infection	Survivors	
					No.†	Per cent.
Penicillin (150×3) *	80	15	2·0	6	59 (25)	73·8
„ (25×3) *	80	36	1·4	16	28 (5)	35·0
„ (25×3) * *plus* anti-toxin	78	18	2·6	8	52 (25)	66·6
Antitoxin alone .	69	43	1·3	3	23	33·3
Untreated controls	80	51	1·1	23	6	7·5

* 150×3 means 150 units for a 20-g. mouse injected subcutaneously at each treatment, etc. Antitoxin dosage as in table II.

† The figure in brackets after no. of survivors shows the number of mice free from abscess formation. No animals in this table are included in table II.

total dosage of 75 units of penicillin for a 20-g. mouse was no more effective than antitoxin alone, while 450 units alone (about one-tenth of the amount tolerated as a single dose) cured over 70 per cent. of the animals. This is a higher proportion of cures than was obtained with maximal dosage of the most effective sulphonamide drugs. Powell and Jamieson (1942) and Robinson (1943) have reached a similar conclusion in comparing penicillin with sulphathiazole. A *striking and important feature* with penicillin treatment was that a large proportion of the survivors showed no abscess formation, in contrast to almost all the survivors in the series receiving sulphonamides and antitoxin alone or together and the few survivors among the untreated controls. It is especially noteworthy that antitoxin *plus* the smaller dose of penicillin gave almost the same result as the large dose of penicillin alone, both as regards the proportion of survivors and freedom from abscesses.

Effect of antitoxin

It is again emphasised that quite irrespective of the ultimate outcome of treatment, whether with sulphonamide drug or penicillin, the animals which received antitoxin were never as ill during the first 6-8 hours after inoculation as those treated with a drug alone. Accordingly, there is an additive effect similar to that previously demonstrated with antitoxin *plus* a sulphonamide drug. This may have a bearing on the treatment of staphylococcal infections in the human subject where toxæmia is a marked feature.

Summary

1. A mouse-virulent strain of *Staphylococcus aureus* suitable for chemotherapeutic trials was maintained by practically continuous alternate mouse passage and culture of the heart blood, the inoculum being given intraperitoneally in mucin. To maintain the virulence of the strain it is important that the organism used should kill a high proportion of mice within 24 hours.

2. In confirmation of our previous work, sulphonamide drugs *plus* antitoxin are in general more effective than drugs alone.

3. When sulphonamide drugs are administered along with antitoxin, sulphamezathine and especially sulphathiazole are more effective than sulphapyridine and sulphanilamide. None of these drugs, however, prevents the development of local lesions at the site of inoculation.

4. Penicillin is more effective than sulphathiazole in respect both of number of survivors and freedom from development of local lesions.

5. The value of combined penicillin and antitoxin therapy has been demonstrated. A small dose of penicillin (relatively ineffective in itself) *plus* antitoxin is as effective as a large dose of penicillin alone.

6. Infected mice which received antitoxin were always in markedly better condition at an early stage than those which received any drug alone.

7. A possible advantage of combined antitoxin and penicillin therapy in man is indicated.

REFERENCES

BARLOW, O. W., AND HOMBURGER, E.   *Proc. Soc. Exp. Biol. and Med.*, 1939, xlii, 792.

BLISS, E. A., AND OTT, E.   .   .   *Ibid.*, 1940, xliii, 706.

BROWNING, P. . . . . . .   This *Journal*, 1940, **l**, 431.

BUTTLE, G. A. H. . . . . . .   *Proc. Roy. Soc. Med.*, 1937-38, xxxi, 154.

DE, S. P., AND BASU, U. P. . .   *Brit. Med. J.*, 1938, ii, 564.

ERCOLI, N., LEWIS, M. N., AND HARKER, E. M.   *Amer. J. Med. Sci.*, 1945, ccix, 621.

FARRELL, L. N. . . . . . .   *Brit. J. Exp. Path.*, 1940, xxi, 302.

FARRELL, L. N., AND KITCHING, J. S.   This *Journal*, 1940, l, 439.

JAWETZ, E. . . . .   *Arch. Int. Med.*, 1946, lxxvii, 1.

KOLMER, J. A., AND BROWN, H. .   *Ibid.*, 1942, lxix, 636.

MILLER, C. P. . . . . . .   *Proc. Soc. Exp. Biol. and Med.*, 1934-35, xxxii, 1136.

POWELL, H. M., AND JAMIESON, W. A.   *J. Indiana Med. Assoc.*, 1942, xxxv, 361.

ROBINSON, H. J. . . . . . .   *J. Pharmacol.*, 1943, lxxvii, 70.

STEINFIELD, L., BRYLAWSKI, M., AND NASH, C. B.   *J. Lab. Clin. Med.*, 1942-43, xxviii, 1544.

THE EFFECTS OF β, β'-DICHLORODIETHYL METHYL-AMINE HYDROCHLORIDE ON THE BLOOD-FORMING TISSUES

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(PLATE LIX)

IN poisoning by mustard gas (dichlorodiethyl sulphide) the blood-forming organs may be damaged with a resulting leucopenia (Stewart, 1918; Cameron, unpublished). During the recent war nitrogenous compounds resembling mustard gas in many of their properties and actions were investigated in England and America. A summary of the investigations made with this group of substances, the so-called "nitrogen mustards", has recently been published in America (Gilman and Philips, 1946).

One of these nitrogen mustards is β, β'-dichlorodiethyl methylamine, which is a vesicant liquid, whereas its hydrochloride is a white, water-soluble crystalline substance. It is the hydrochloride which has been used in these investigations. It was shown both by English and American investigators that this substance has a consistent action on the lymph glands, spleen and bone marrow, causing a leucopenia. American investigators have also used it therapeutically in man in cases of Hodgkin's disease, leukæmia and new-growths (Rhoads, 1946).

The experiments here reported deal with the changes occurring in the blood-forming tissues, the blood-cell counts and the production of blood cells after administration of this nitrogen mustard in rabbits and dogs.

METHODS

In the experiments in which the thoracic duct was cannulated in dogs, the animals were anæsthetised with Nembutal. Otherwise all animals were unanæsthetised. Blood samples were taken from the ear vein in rabbits and from the jugular vein in dogs. Blood counts were made in the usual way. For differential white cell counts 500 or 1000 cells were counted, except when the white cell count was extremely low, in which case four blood films had to be searched to count 50 white cells. Sections of the blood-forming tissues were stained with Ehrlich's acid hæmatoxylin and eosin, Weigert's iron hæmatoxylin and van Gieson, and Giemsa and Gömöri's reticulin method.

The β, β'-dichlorodiethyl methylamine hydrochloride was always freshly made up in distilled water just before injection and was given either intravenously or subcutaneously, the response being similar by either route.

RESULTS

Histological changes in the blood-forming organs

Lymph glands. With doses of 2 and 3 mg./kg. body weight, progressive necrosis of the germinal centres with infiltration of polymorphs commences as early as 3-6 hrs. after administration in both rabbit and dog (fig. 1). The necrotic debris is cleared away by 24 hrs., when larger reticulo-endothelial cells occupy the middle of the germinal centres. If the animal survives, the germinal centres appear normal again by the 9th day, the large number of polymorphs gradually disappearing. Similar changes occur with doses of 1 mg./kg., but the depletion of the germinal centres is less.

Spleen. The changes closely resemble those seen in the lymph glands (fig. 2).

A neat demonstration of the early onset of splenic injury is possible with intra-ocular splenic grafts in rabbits. Four to 6 weeks prior to the injection experiment, tiny pieces of the animal's own spleen are inserted into the anterior chamber of the eye where they often grow into miniature spleens. If such grafts are watched with a magnifying glass and suitable illumination, they are seen to change colour from red to yellow 2-3 hrs. after nitrogen mustard is injected intravenously. Occasionally the colour change sets in within an hour of injection. Microscopical signs of necrosis appear shortly afterwards.

Bone marrow. With doses of 2 and 3 mg./kg. there is no obvious change during the first 5 hrs. except some congestion of the sinusoids. From 24 to 72 hrs. there is a rapid depletion of the myeloid tissue, the sinusoids becoming congested and the stroma oedematous (fig. 3). New haematopoietic foci appear after a few days in animals which survive (fig. 4), and regeneration is complete in about 1-3 weeks.

The red and white cells in the blood

Effect of a single dose. Unanæsthetised rabbits and dogs were used in these experiments. In the normal dog the lymphocyte count is much less than the granulocyte count and eosinophils are much more numerous than basophils. In the rabbit the lymphocyte count is usually greater than the granulocyte count and there are more basophils than eosinophils. Despite these normal differences the effect of β, β'-dichlorodiethyl methylamine hydrochloride was similar in the two animals. The blood of 32 rabbits was investigated before and at varying times after the administration of 1, 2 and 3 mg./kg. In all cases the results were fairly consistent; the larger the dose, the greater the fall in the white cell count. Typical results are shown in

Fig. 1.—Mesenteric lymph node from dog receiving 3 mg. per kilo of nitrogen mustard. Killed after 5 hours. Extensive necrosis in a follicle. H. and E. ×270
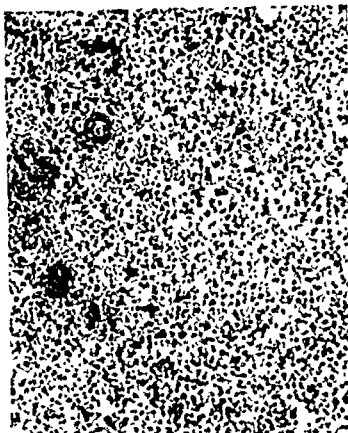
Fig. 2.—Spleen from dog receiving 3 mg. per kilo nitrogen mustard. Killed after 24 hours. Extensive necrosis in malpighian body and pulp. H. and E. ×270.
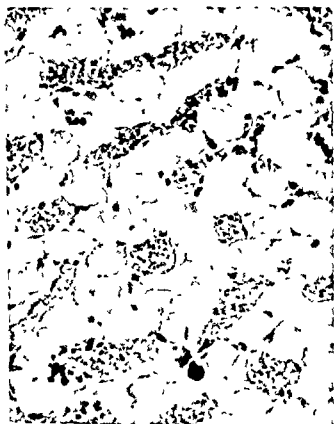
Fig. 3.—Bone marrow from rabbit receiving 3 mg. per kilo nitrogen mustard. Killed on second day. Depletion of hæmopoietic tissue. Necrosis of many cells; œdema and shrinking of fat cells. H. and E. ×270.
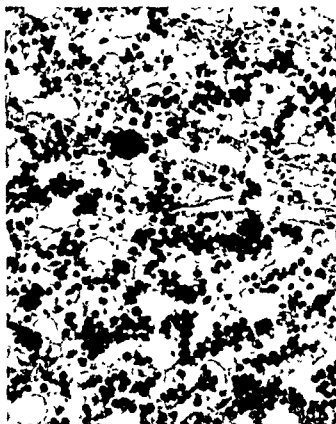
Fig. 4.—Bone marrow from rabbit receiving 1 mg. per kilo nitrogen mustard. Killed on ninth day. Hæmopoiesis proceeding actively. H. and E. ×360.

table I, which gives the mean white-cell counts in a group of 5 rabbits before and after the intravenous administration of 1 mg./kg., and

TABLE I

Blood white-cell counts, thousands per c.mm., before and after injections of β, β'-dichlorodiethyl methylamine hydrochloride

(a) Mean of 5 rabbits, 1 mg./kg. intravenously, unanæsthetised				
	Total	Neutrophils	Lymphocytes	Basophils
Before injection .	10·6	5·13	5·36	0·15
5 hrs. after injection .	10·3	7·21	2·89	0·17
1 day ,, ,, .	6·0	4·64	1·29	0·06
2 days ,, ,, .	3·1	1·68	1·38	0·01
3 ,, ,, ,, .	1·1	0·07	1·06	0·01
4 ,, ,, ,, .	0·45	0·01	0·44	0·00
5 ,, ,, ,, .	2·8	0·16	2·61	0·02
7 ,, ,, ,, .	9·1	6·07	2·87	0·13

(b) Mean of 5 dogs, 2 mg./kg. subcutaneously, unanæsthetised				
	Total	Neutrophils	Lymphocytes	Eosinophils
Before injection .	12·8	8·11	2·83	1·86
1 hr. after injection .	13·4	9·00	2·91	1·49
2 hrs. ,, ,, .	15·5	11·94	1·88	1·69
3 ,, ,, ,, .	18·0	14·97	1·88	1·11
4 ,, ,, ,, .	19·5	17·56	1·34	0·55
5 ,, ,, ,, .	22·5	20·73	1·34	0·38
24 ,, ,, ,, .	16·8	16·50	0·30	0·00

the mean counts of a group of 5 dogs before and after the subcutaneous injection of 2 mg./kg. In the case of the dogs, changes later than 24 hrs. are shown in fig. 6 (p. 431).

In rabbits the neutrophils at first showed a considerable increase, beginning 1 hr. after administration. After this initial rise, which might last 24 hrs., the neutrophil count fell to reach a minimum on the 3rd or 4th day. As seen from the table the count might reach almost zero, and yet the animal might survive. If the animal survived, the count rapidly rose on the 5th day and was usually back to normal on the 7th day. The lymphocyte count fell from the beginning. In 5 hrs., as seen in table I, it had fallen considerably and continued to fall until it reached a minimum on the 3rd or 4th day. From the 5th day onwards the lymphocytes rose rapidly to normal. The basophils in rabbits and the eosinophils in dogs behaved in a similar manner to the lymphocytes and practically disappeared from the blood. They returned with the recovery of the other white cells.

Effect of repeated doses in rabbits. In the above experiments the fall and subsequent rise in all elements of the white-cell count were

so rapid that the red-cell count was not much altered, even though the bone marrow was considerably damaged for a short time. Repeated doses were therefore given to keep the white-cell count low for a period of about 4 weeks and the effect on the red-cell count observed.
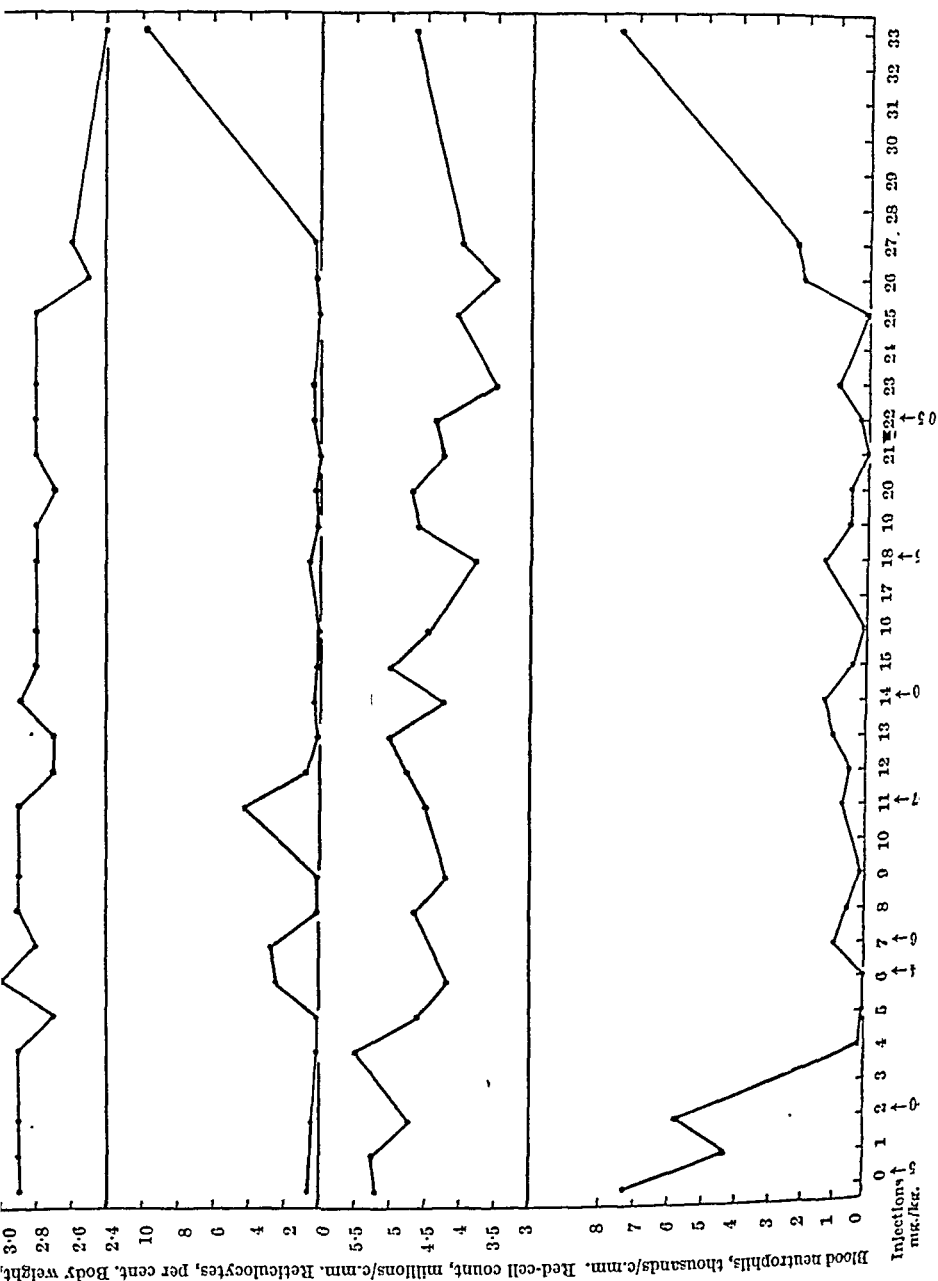
In these experiments rabbits were used. Varying doses of β, β'-dichlorodiethyl methylamine hydrochloride were given at intervals when it was thought that the white-cell count was about to rise. In

this way all white cells were kept at a low level continuously for about 4 weeks, daily counts being made. The results of a typical experiment are shown in fig. 5. In these experiments, the red-cell count fell only gradually, even though the neutrophil count was daily below 1000, indicating considerable damage to the bone marrow for 4 weeks. At the end of this time, when the injections ceased, the white-cell count rapidly rose, accompanied by a reticulocyte response and a rise in the red-cell count.

These results suggest that the production of both neutrophils and red cells in the bone marrow is greatly reduced, but the life of the circulating red cell is much longer than that of the neutrophil.

The production of the blood cells after the administration of β, β'-dichlorodiethyl methylamine hydrochloride

Lymphocytes. Dogs were used in this investigation. For the collection of lymph, the dogs were anæsthetised with Nembutal. The thoracic duct was cannulated and the lymph collected in hourly periods for 5 hrs. White-cell counts were made on each sample of lymph. At the same time hourly samples of blood were taken for white-cell counts. This procedure was carried out in normal dogs and in dogs 1, 2 and 3 days after the administration of β, β'-dichloro-diethyl methylamine hydrochloride. In these last three groups, the substance was administered in the unanæsthetised animals and the blood counts followed for the stated time. Then Nembutal was given, the thoracic duct cannulated and the lymph collected. Thus only the lymphocyte production in the drainage area of the thoracic duct was determined, but this is probably by far the greatest source of lymphocytes in the blood. The thoracic duct was cannulated in the morning with the dog in the post-absorptive condition. No transfusions were given to increase the lymph flow.

Effect of Nembutal anæsthesia on the white-cell count in dogs. As the lymph was collected under Nembutal anæsthesia in all cases, control experiments were made to ascertain the effects of this anæsthetic on the white-cell count in the blood. In table II are shown the changes in the white-cell counts of the blood in 5 dogs under Nembutal anæsthesia for 5 hrs. No operation was performed except venepuncture of the jugular vein for samples of blood every hour. The neutrophil, lymphocyte and eosinophil counts all fell somewhat after Nembutal.

However, if the operation of cannulating the thoracic duct is performed, the white-cell picture of the blood is as shown in fig. 6 (a). The operation consists of an incision in the lower part of the left side of the neck and the clearing of tissue to enable the thoracic duct to be cannulated just before it enters the vein. The neutrophil count shows an initial fall, followed, during the succeeding 5 hrs., by a very considerable rise. During the 5 hrs. that the thoracic duct

lymph was collected, the lymphocyte count of the blood decreased, as the majority of lymphocytes were not entering the blood stream.

TABLE II

Effects of Nembutal anæsthesia for 5 hrs. on white cell counts, thousands per c.mm., in normal dogs. Mean of 5. No operation

	Total	Neutrophils	Lymphocytes	Eosinophils
Before Nembutal .	11·56	8·22	2·21	1·13
¼ hr. after Nembutal	9·88	6·83	2·22	0·83
1 ,, ,, ,,	9·25	6·73	1·49	1·03
2 hrs. ,, ,,	9·18	6·70	1·54	0·94
4 ,, ,, ,,	9·12	6·92	1·35	0·85
5 ,, ,, ,,	10·07	7·84	1·48	0·75

The fall was not steady, however, and towards the end a slight rise was usually observed. The fall in the eosinophil count was continuous, and at the end of 5 hrs. the eosinophils had almost disappeared from the circulating blood. Where they go is not known.

Lymphocyte production in normal dogs and in dogs 1, 2 and 3 days after the injection of 2 mg. of nitrogen mustard per kg. The average results of these experiments are shown graphically in fig. 6. Fig. 6 (a) represents the mean results in 5 normal dogs. The lymph flow and lymphocyte count of the lymph varied somewhat in individual dogs, but this variation did not compare with the changes seen after the administration of β, β′-dichlorodiethyl methylamine hydrochloride.

Fig. 6 (b) depicts the mean results of experiments on 4 dogs one day after injection. The blood lymphocyte count had fallen from 2500 to 530, the eosinophil count from 1200 to 90, while the neutrophil count had risen from 7600 to 16,500 before Nembutal was given. After collecting the lymph for 5 hrs. the lymphocytes and eosinophils fell further, while the neutrophils rose somewhat. This rise, however, was not as great as in normal animals. The lymph flow in these animals was reduced to less than half that of normal dogs, while the lymphocyte count in the lymph had also fallen considerably. Thus the output of lymphocytes from the lymph glands was very greatly decreased.

Fig. 6 (c) depicts the mean results of experiments on 4 dogs two days after injection. At this time the blood lymphocytes and eosinophils were at an even lower level than in the previous experiments. The lymph flow was about half normal and the lymphocyte count in the lymph was further reduced. The output of lymphocytes was thus still greatly reduced on the second day. The neutrophil count in the blood was about the same at the end of two days as the normal count, having first risen and then fallen. When the animal was anæsthetised and the thoracic duct cannulated, the neutrophil count

fell further and did not show the neutrophilia observed in normal
dogs. This suggests that the damaged bone marrow cannot respond
to the stimulus of trauma.
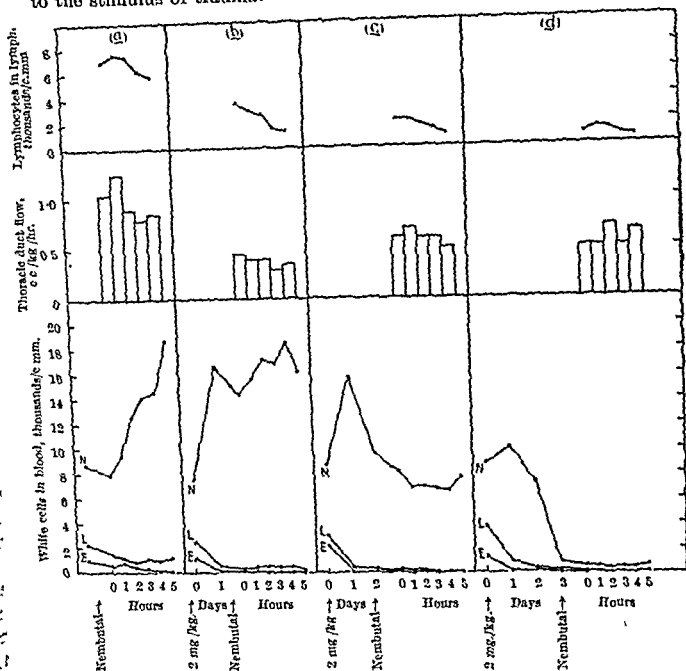
Fig. 6.—Effect of β, β'-dichlorodiethyl methylamine hydrochloride, 2 mg./kg. sub-
cutaneously, on the white-cell count (N = neutrophils, L = lymphocytes, E =
eosinophils), thoracic duct lymph flow and lymphocyte content of lymph in dogs.

(a) Mean of 5 normal dogs.
(b) Mean of 4 dogs 1 day after injection.
(c) Mean of 4 dogs 2 days ,,   ,,
(d) Mean of 4 dogs 3 ,,   ,,   ,,

Fig. 6 (d) shows the mean results of experiments on 4 dogs three
days after injection. On the 3rd day there were very few white cells
in the blood—600/c.mm. in these experiments. The lymphocytes
and eosinophils had practically disappeared. Cannulation of the
thoracic duct gave no neutrophil response. The lymph flow was
still about half normal, but the lymphocyte count was very low,
so that the output of lymphocytes by the lymph glands was now
very greatly reduced.

The total number of lymphocytes in the blood—assuming a blood volume of 79 c.c./kg. (Courtice, 1943-44) just before lymph collection —and the production of lymphocytes in these experiments are summarised in table III. This table shows how the blood lymphocytes

TABLE III

The lymphocytes in the blood and thoracic-duct lymph in dogs before and after the subcutaneous injection of β, β'-dichlorodiethyl methylamine hydrochloride, 2 mg./kg. Dogs under Nembutal anæsthesia. Figures in brackets denote number of animals in each group

	Lymphocytes in blood at beginning of lymph collection (millions per kg. body weight)	Lymphocytes in lymph. Mean of 5 hours collection. (Millions/kg./hr.)
Controls (5) . . .	108	6·7
1 day after injection (4) .	25	2·2
2 days „ „ (4) .	12	2·7
3 „ „ „ (4) .	4	0·9

and the production of lymphocytes fell with progressive damage to the lymph tissue.

Production of red cells after β, β'-dichlorodiethyl methylamine hydrochloride. It was observed that after a single injection, the white-cell count might fall to an extremely low level for a few days with temporary damage to the bone marrow. The red-cell count, however, did not alter much. During repeated doses the neutrophil count was kept low for 4 weeks, with a gradual fall in red-cell count from about $5\frac{1}{2}$ to 4 million. This suggests that the normal replacement of red cells had been much decreased.

To demonstrate that red-cell production is reduced when the bone marrow is damaged, the reticulocyte response to hæmorrhage in normal rabbits and in rabbits in which the neutrophil count had been kept low was investigated. In a group of normal rabbits, red-cell and reticulocyte counts were made daily before and after two hæmorrhages of 14 c.c./kg., one on each of two consecutive days. In another group of rabbits the neutrophil count was kept low for several days by repeated injections of β, β'-dichlorodiethyl methylamine hydrochloride. During this period of leucopenia, these animals were bled on 2 consecutive days to the same extent as the control group. The results are shown in fig. 7. Following the hæmorrhages, the red-cell count in the control group fell from 5·4 to 2·9 million per c.mm., while that of the second group fell from 5·3 to 2·9 millions per c.mm. Thus the degree of anæmia was almost identical in each group. In the control group there was a sharp reticulocyte response on the second day after the second hæmorrhage, reaching a peak on the third day. In the other group the reticulocyte response was delayed, only rising to a considerable height when the injections were discontinued. The rapid rise in the reticulocytes corresponded to the rise in the neutrophil count. The results in individual animals

were similar as far as the time factor was concerned, so the average figures give a true picture of the course of events. These experiments
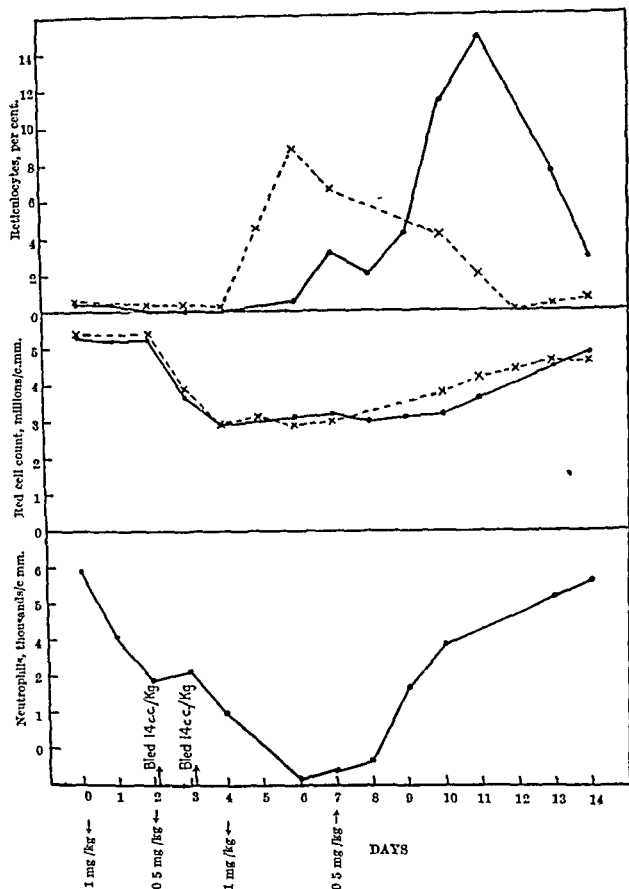
Fig. 7.—The reticulocyte response to hæmorrhages, 14 c.c./kg. on each of 2 successive days, in normal rabbits (x – – – – x) and in rabbits after repeated injections of β, β'-dichlorodiethyl methylamine hydrochloride (●————●).

suggest that when the bone marrow is damaged and the neutrophil count is low, the production of red cells is much reduced. This leads to anæmia if the lesion is maintained for a prolonged period.

DISCUSSION

The action of this nitrogen mustard on the blood-forming organs has been shown to be very rapid, with an equally rapid recovery. The fall in the white-cell count of the blood is greater and quicker than that of the red-cell count, because the life of the circulating white cells is very short compared with that of the red cells. Thus as soon as the production of white cells is reduced by the action of β, β'-dichlorodiethyl methylamine hydrochloride on the lymphatic tissue and bone marrow, the blood white-cell count falls suddenly to a low level. The red-cell count on the other hand is not so much altered, even though fewer new red cells are being formed. In all the animals that survived, the white-cell count rapidly recovered when the injections ceased, in spite of quite considerable damage to the blood-forming tissues for longer or shorter periods. The blood-forming tissue thus has a truly remarkable power of recovery.

This action of the nitrogen mustards resembles that of X-rays. For this reason their effects on such diseases as Hodgkin's disease, leukæmia and new-growth have been investigated (Rhoads). One of the disadvantages of their therapeutic use is the generalised damage to all the blood-forming tissues. In lymphatic leukæmia or Hodgkin's disease, for example, not only the lymph glands but also the bone marrow will be affected. Another disadvantage is the transient nature of the damage. Even if repeated injections are given for 4 weeks in normal animals, recovery is complete a few days after the cessation of these injections. A further disadvantage is the gradual anæmia which is produced by prolonged bone-marrow damage. This, however, can be corrected by blood transfusions. In several experiments with rabbits, after β, β'-dichlorodiethyl methylamine hydrochloride, blood transfusions increased the red-cell count considerably but had no effect on the low white-cell count.

SUMMARY

The effects of one of the nitrogen mustards, β, β'-dichlorodiethyl methylamine hydrochloride, on the blood-forming organs have been investigated in rabbits and dogs.

There is progressive necrosis of the germinal centres of the lymph glands and spleen, with polymorphonuclear infiltration, commencing as early as 3 hrs. after injection and progressing until the 3rd or 4th day. The bone marrow shows at first congestion of the sinusoids and then a rapid depletion of the myeloid tissue from 1 to 3 days after injection. Recovery occurs in about a week.

The white cells of the blood are affected. A neutrophilia is observed during the first 24 hrs., followed by a neutropenia. The lymphocytes, basophils and eosinophils begin to fall within a few hours after injection. The fall in all cells is at a maximum on the 3rd or 4th day, and from the 5th to the 7th day all cells return to normal if the animal survives.

The red cells are little affected by a single dose, but repeated injections for 4 weeks cause progressive anæmia.

Collection of the thoracic-duct lymph in dogs shows that lymphocyte production is greatly reduced as the blood lymphocyte count falls. The production of neutrophils is also greatly decreased, as there is no neutrophil response to trauma when the bone marrow is damaged. The formation of new red cells is likewise decreased, as the normal reticulocyte response to hæmorrhage is not observed when the neutrophil count is kept low by repeated injections, but is evident as soon as the neutrophils rise on cessation of the injections.

REFERENCES

COURTICE, F. C. . . . . . 1943-44. *J. Physiol.*, cii, 290.

GILMAN, A., AND PHILIPS, F. S. . 1946. *Science*, ciii, 409.

RHOADS, C. P. . . . . . . 1946. *J. Amer. Med. Assoc.*, cxxxi, 656.

STEWART, M. J. . . . . 1918. Medical Research Council, Reports of the Chemical Warfare Medical Committee, no. 17, *London*.

THE MYOEPITHELIUM IN CERTAIN TUMOURS OF THE BREAST

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(PLATES LX-LXIV)

MIXED epithelial and mesodermal tumours are rare, though some tissues, particularly sweat glands and salivary glands, often give rise to tumours of mixed appearance. In these, the stroma probably arises from the proliferation of a special layer of cells, the so-called myoepithelium (Hamperl, 1931; Sheldon, 1941, 1943). These cells, which develop from the embryonic epithelium (Peyron, Corsy and Surmont, 1926), lie outside the epithelium typical of the gland and often acquire the form and staining reactions of smooth muscle. Tumours derived from both epithelium and myoepithelium may appear " mixed " but they are wholly epithelial in origin.

von Kölliker (1848-49) first described the myoepithelium when he found smooth muscle cells within the basement membrane of sweat glands and apocrine glands. Later, similar cells were described in the breast, salivary glands and mucous glands of the palate. In the breast the myoepithelium is discernible between the 7th and 8th months of intra-uterine life. Initially the cells are rounded and may remain so, or they may become spindle-shaped, often with the staining reactions of smooth muscle. In animals, particularly the bitch, mixed tumours of the breast are common ; in these, as in the mixed salivary and sweat gland tumours of man, the complex stroma arises from the myoepithelium (Peyron, 1924 ; Peyron, Corsy and Surmount, 1926 ; Peyron and Montpellier, 1932 ; Hamperl, 1939-40). Hamperl (1939-40) found mixed epithelial and myoepithelial proliferation in human mastopathy and suggested that some mixed tumours of the breast might be partly myoepithelial. Durante and Roulland (1921) and Gaudier, Grandclaude and Lambert (1931) described human breast tumours in which the myoepithelium was probably involved.

The purpose of the present study is to compare the myoepithelial

proliferation which was found in certain human breast tumours with that which occurs in breast tumours of the bitch.

The specimens were fixed in 4 per cent. formol-saline. Paraffin sections were stained with hæmatoxylin and eosin, Masson's trichrome stain and a modification of Foot's reticulin impregnation.

TUMOURS OF THE BREAST IN THE BITCH

In these tumours, glandular epithelium occurs in a stroma which may contain smooth muscle, myxomatous material, cartilage and bone. Nine mixed tumours were examined. It is not proposed to describe these in detail since their study confirmed the findings of previous writers.

In most of the tumours, an epithelial type of reticulin pattern is present (fig. 4). Epithelial acini and masses of proliferated myo-epithelial cells are sharply delimited (fig. 1), but in parts of the more complex tumours reticulin, collagen, cartilage and bone may be deposited in what, from its general structure, appears to be a myo-epithelial stroma (figs. 2 and 7). In some places it is not difficult to trace a transition from groups of myoepithelial cells to isolated cells morphologically indistinguishable from chondrocytes (fig. 3). The individual myoepithelial cells are either rounded and vacuolate or spindle-shaped, often with the staining reactions of smooth muscle.

In one animal the breast contained three hard masses which show a progressive increase in reticulin formation. In one tumour there is a clearly glandular pattern with marked myoepithelial proliferation (fig. 4); in the second, areas of spindle cells, mainly with the staining reactions of smooth muscle, are delimited by reticulin outlines, but fine strands of reticulin occur also between the individual cells (fig. 5). In a lymph-node metastasis traces of the original pattern remain, but the fine reticulin network is so prominent that histologically the structure is that of a sarcoma (fig. 6). In a case of this sort it is impossible to be certain that one is not dealing with several completely different types of tumour, but the transitions of structure shown suggest that they may all be of epithelial and myoepithelial origin.

The unusual structure of these tumours makes it difficult to assess the degree of malignancy. Three of the nine were malignant by ordinary histological criteria. The remaining six had a highly complex structure and, since the clinical course was not followed, it is impossible to be certain whether they should be considered benign or malignant.

TUMOURS OF THE HUMAN BREAST

The structure of the human tumours is seldom as complex as those of the bitch. Nevertheless there is often myoepithelial proliferation

Fig 1.—Mixed tumour of bitch showing a single acinus of deeply staining epithelial cells and loosely arranged myoepithelial cells sharply differentiated from the surrounding stroma (R I S H 498/45) ×110

Fig 2.—Mixed tumour of bitch. Lower half, Masson's trichrome stain; upper half, reticulin impregnation. The lower part shows masses of myoepithelial cells and some deeply staining epithelial acini. In the upper part the formation of reticulin between the myoepithelial cells may be seen. (R I S H 914/45) ×110.

Fig 3.—Mixed tumour of bitch. In the upper part are small groups of myoepithelial cells, a transition can be traced between these and cells isolated by a hyaline stroma in the lower part. Masson's trichrome stain. (R I S H 497/45) ×260.

Fig 4.—Bitch tumour. Reticulin impregnation showing glandular pattern (R I S H. 496/45) ×110

MYOEPITHELIUM IN BREAST TUMOURS

FIG. 5.—Second tumour from same animal as fig. 4. Reticulin impregnation showing some evidence of glandular pattern but also the formation of reticulin between the individual myoepithelial cells. (R.I.S.H. 496/45.) ×110.
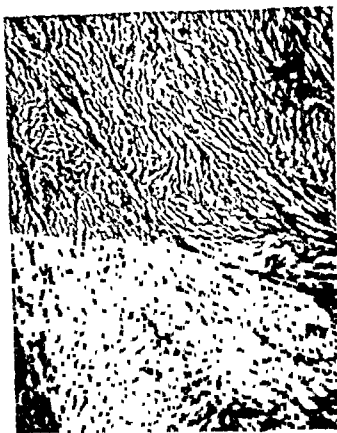
FIG. 6.—Lymph-node metastasis from the same animal as figs. 4 and 5. Reticulin impregnation to show sarcomatous pattern. (R.I.S.H. 496/45.) ×110.

FIG. 7.—Mixed tumour of bitch showing cartilage formation. (R.I.S.H. 934/45.) ×70

FIG. 8.—Chronic mastopathy. In the upper part are masses of proliferated epithelial and myoepithelial cells and in the lower part there is a bundle of smooth muscle cells in the stroma. Masson's trichrome stain. (R.I.S.H. 548/45.) ×110.

FIG 9 —Chronic mastopathy Upper left, masses of proliferated epithelial and myoepithelial cells Lower right, dense fibrous stroma in which individual spindle shaped myoepithelial cells may be discerned Masson's trichrome stain (R I S H 1517/45) ×110

FIG 10 —Mastopathic nodule in the human breast showing myoepithelial proliferation and small groups of myoepithelial cells in the stroma (R I S H 3201/46) ×300

FIG 11 —Intraduct papilloma of the human breast showing loosely arranged myoepithelial layer (R I S H 1785/45) ×75

FIG 12 —Single acinus from intraduct papilloma shown in fig 11 Deeply staining epithelium and pale staining myoepithelium are shown Immediately above this there is a horizontally arranged myoepithelial cell with the staining reactions of smooth muscle and showing longitudinal fibrils in the cytoplasm (R I S H 1785/45) ×510

MYOEPITHELIUM IN BREAST TUMOURS

FIG. 13.—Intraduct papilloma of the breast shown in figs. 11 and 12. Less regularly arranged myoepithelial cells. (R.I.S.H.) ×300.
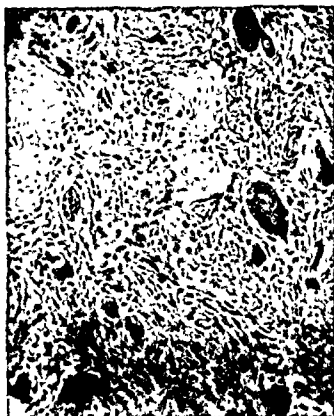
FIG. 14.—Case 1. Mixed tumour of the human breast showing small epithelial cell masses, cellular stroma and bone formation. (R.I.S.H. 1307/44.) ×110.

FIG. 15.—Case 1. Showing masses of irregular epithelial and myoepithelial cells. (R.I.S.H. 1307/44.) ×110.

FIG. 16.—Case 1. Showing a mass of (?) myoepithelial cells merging with the surrounding cellular stroma. (R.I.S.H. 1307/44.) ×110.

and some suggestion that myoepithelial cells may play a part in the formation of the stroma. The tumours studied were :—

    I. Proliferative mastopathy and intraduct papilloma.

    II. Mixed tumours.

    III. Malignant tumours.

    IV. Tumours classified as Brodie's sero-cystic disease of the breast.

Proliferative mastopathy and intraduct papilloma

In mastopathic nodules and intraduct papillomata it is not uncommon to find some myoepithelial proliferation. Of one hundred specimens of mastopathy examined, twenty-five showed moderate and four marked myoepithelial overgrowth. This hyperplasia has been described by Günther (1937), Hamperl (1939-40) and Kuzma (1943). All these authors emphasise the fact that although the hyperplasia is benign, it may simulate carcinoma.

Three examples of mastopathic myoepithelial proliferation are illustrated in figs. 8-10. In all these there is some difficulty in distinguishing myoepithelial cells from stroma. In one case (fig. 8) the stroma contains smooth muscle which may be myoepithelial, though this is difficult to prove, for a smooth-muscle cell once detached from a glandular acinus has no characters by which it may be differentiated from a similar cell of mesodermal origin.

The type of myoepithelial proliferation common in intraduct papillomata is shown in figs. 11-13. In some areas the myoepithelial cells, which are either vacuolate or spindle-shaped with the staining reactions of smooth muscle, form a clearly defined layer ; in others the layer is several cells in thickness.

Mixed tumours of the human breast

In comparison with the bitch, mixed tumours of the human breast are rare. In a review of the literature from 1860 to 1945 Rottino and Willson (1945) record 78 cartilaginous and bony tumours. These include 7 enchondromata, 21 chondrosarcomata, 8 osteochondromata, 12 osteochondrosarcomata, 5 osteosarcomata and 25 giant-cell tumours. Other mixed tumours include an adenomatous tumour with smooth muscle in the stroma described by Abramow (1901), two carcinosarcomata with a leiomyomatous stroma described by Hamperl (1939-40) and carcinomata with a spindle-celled stroma described by Hertzler and Koeneke (1933) and Tudhope (1939). In addition benign leiomyomata were described by Strong (1913), Melnick (1932) and Stein (1942).

The three tumours examined in the present series all showed glandular epithelium in stroma containing smooth muscle, cartilage or bone.

Case 1

The patient, aged 76, had noticed a mass in the left breast for six months. On admission the left breast, which was larger than the right, was wholly replaced by a hard mass with cystic areas. Since this was not fixed to the pectoral muscle and axillary lymph nodes were not palpable, it was removed by local mastectomy. The patient made an uneventful recovery and was without evidence of recurrence one year later.

Macroscopically the tumour (R.I.S.H. 1307/44) was a hard encapsulated mass measuring about 8 cm. in diameter. On section, it contained two main cystic spaces and was of an unusually firm, rather gritty consistency, with areas resembling cartilage.

Microscopically, the stroma is made up in part of densely aggregated spindle cells and collagen and in part of a loose network of cells with little intercellular substance. There is some hyaline change in the stroma but no true cartilage. Small areas of bone are numerous and giant cells are widely distributed, some being applied to the bony masses like osteoclasts (fig. 14). The cystic spaces are lined by epithelium and epithelial masses occur throughout the stroma. Some of these show squamous metaplasia (fig. 15) and in some the cells are spindle-shaped, with the staining reactions of smooth muscle. A myoepithelial layer is usually present, and in some cases this is closely applied to bone. It is often difficult to draw a sharp distinction between the myoepithelium and the stroma, because strands of cells apparently arising from the myoepithelium merge with the stroma and the cells are morphologically similar (fig. 16).

Case 2

A woman aged 65 had noticed for seven months that her right breast was enlarged. It increased rapidly in size and on admission was five or six times larger than the left. No axillary lymph nodes were palpable. The growth was removed by local mastectomy and the patient made a good recovery. One year later there was no recurrence.

Macroscopically the tumour (R.I.S.H. 3004/44) was a hard encapsulated mass measuring $22 \times 20 \times 13$ cm. On section it was solid, of greyish white colour and divided into coarse lobules by clefts, the lining of which was smooth and shining.

Microscopically the stroma is composed of large spindle cells, mainly with the staining reactions of smooth muscle, in a framework of collagen fibrils. There is some hyaline change in the collagen, but neither cartilage nor bone is present. Scattered throughout are regular tubules lined by epithelium which is composed of two layers, an inner of deeply staining cells and an outer of myoepithelium, mainly of rounded vacuolated cells. In some places these cells have the staining reactions of smooth muscle, in others there is proliferation of epithelial type, sometimes with squamous metaplasia. Similar epithelium lines the larger clefts. In one or two places there is a tendency for myoepithelial cells to break through the basement membrane and mingle with the stroma (fig. 17).

Case 3

The patient was 68. Six years previously she had had a right radical mastectomy. Four years later a nodule was excised from the scar. On admission to St Bartholomew's Hospital there was a further recurrence of six months' duration. The mass, which was about three inches in diameter, was adherent to both skin and chest wall. The tumour was removed together with portions of the fourth and fifth ribs and an area of pleura to which it was adherent. Convalescence was complicated by a right pneumothorax, but the patient recovered and was well until seven years later, when she died with a fourth local recurrence.

Macroscopically the tumour (St Bartholomew's Hospital S.H. 9576) measured 7 × 5 cm. and was of firm consistency. On section it was solid, had a whorled appearance and in places appeared to be undergoing mucoid degeneration.

Microscopically the stroma is composed of spindle cells and collagen, with areas of cartilage and bone formation. The glandular epithelium is scanty but that present shows both epithelial and vacuolate myoepithelial layers.

In these cases as in the bitch tumours it is difficult to assess the degree of malignancy. In case 3 the histological structure does not suggest any special invasive power and yet the tumour was persistently recurrent. In case 1 there are irregular masses of glandular epithelium suggestive of malignancy, yet the tumour as a whole was encapsulated. In case 2 the tumour is histologically benign. Neither of these cases showed recurrence within a year.

These tumours differ in structure from those of the bitch and indeed there is no evidence that human myoepithelium has the same tendency to excessive proliferation and differentiation. However, in case 1 there is some evidence that myoepithelial cells may contribute to the stroma and bone is formed in close association with myoepithelial cells. In case 2 the occasional penetration of myoepithelial cells into the stroma suggests that the stromal smooth muscle may be myoepithelial. There is, however, no proof of this and the smooth muscle might be derived from the walls of blood vessels or even from the smooth muscle of the subcutaneous tissue (the arrectores pilorum).

Malignant tumours

There is seldom good evidence that the myoepithelium plays any part in the development of malignant tumours of the breast. In the carcinoma with a spindle-celled stroma described by Tudhope (1939) the stroma may have been myoepithelial and in the tumours described by Hertzler and Koeneke (1933) as " circumscribed carcinoma of the mammary gland " the myoepithelium may have been involved, though in neither case was the staining reaction of smooth muscle observed. Hamperl (1939–40) reported two carcinomata with a leiomyomatous stroma in which, as he suggested, the smooth muscle may have been derived from myoepithelium. Malignant myoepithelial tumours were reported by Gaudier *et al.* and Durante and Roulland. In the two cases here recorded there is some suggestion that the myoepithelium may have taken part in the tumour formation.

Case 4

The patient, aged 34, had noticed a nodule in the left breast for six weeks. This was removed locally. A year later, a swelling was noticed at the site of the first operation. This was again excised and four weeks later there was a firm tumour measuring 3 × 2 inches immediately deep to the two scars. No axillary lymph nodes were palpable. The breast was removed by local mastectomy.

The nodule removed at the first operation (Salisbury General Hospital S.H. 388/44) is composed of mastopathic tissue with an unusually cellular stroma. In one area there is marked myoepithelial proliferation with no very clear differentiation between the myoepithelium and the surrounding stroma. The specimen from the second removal (Salisbury General Hospital S.H. 458/45) consisted of fragments of soft tumour tissue. These are made up of spindle cells with areas of hæmorrhage and necrosis. The structure is similar to that of the third specimen. The local mastectomy specimen (R.I.S.H. 3044/46) contained a yellowish-white soft circumscribed tumour about 3 cm. in diameter, showing areas of hæmorrhage and necrosis. In the mastopathic tissue, of which the breast tissue was composed, were small nodules of similar appearance. Microscopically this tumour is made up of spindle and stellate cells. Some of these have the staining reactions though not the general appearance of smooth-muscle cells (fig. 18). In some areas reticulin impregnation shows a diffuse pattern of sarcomatous type ; in others, masses of cells are outlined by reticulin in the manner typical of epithelial tumours (fig. 19).

The structure of this tumour may be interpreted in either of the two ways. It may be a sarcoma arising from the stroma of the first specimen or it may be a myoepithelial tumour arising from the proliferated myoepithelium in the first specimen. In favour of the first hypothesis is the marked cellularity of the stroma in the first specimen. In favour of the second are the myoepithelial proliferation of the first specimen, the epithelial type of reticulin pattern that occurs in parts of the main tumour and the occurrence of cells with the staining reactions of smooth muscle.

Case 5

The patient, aged 56, had noticed a lump in the left breast for fifteen months. On admission the left breast, which was smaller than the right, was replaced by a hard nodular mass fixed to the nipple and causing retraction. A hard lymph node was palpable in the left axilla. The tumour was removed locally and post-operatively the patient was given X-ray treatment. Macroscopically the breast tissue of the specimen (R.I.S.H. 3153/46) was largely replaced by a hard carcinoma.

Microscopically most of the tumour consists of widely infiltrating spheroidal-celled carcinoma. In one area there is a nodule about 0·5 cm. in diameter of different structure. In this there are well formed glandular acini with a myoepithelial layer of both vacuolate cells and spindle cells with the staining reactions of smooth muscle. In the stroma are areas of cartilage. A transition can be traced between groups of vacuolate myoepithelial cells and cells isolated by the cartilaginous matrix (fig. 20). It seems probable that the cartilaginous stroma in this nodule was derived from the myoepithelium.

Brodie's sero-cystic disease of the breast

In 1846 Brodie described a type of breast tumour of large size and rapid growth, characteristically cystic and often with intra-cystic papillomata. These tumours did not metastasise but were liable to local recurrence. Histological sections from case 6 in Brodie's original paper (St George's Hospital Museum, no. 28 in the Historical Section) showed the reticulin pattern of a fibroadenoma.

Subsequent accounts of these large fibroadenomata have shown a confusion of nomenclature. By some they are called soft fibro-adenomata, by some adenosarcomata (though histologically there are no means of distinguishing the tumours which are most liable to

MYOEPITHELIUM IN BREAST TUMOURS

FIG. 17.—Case 2. Tubule lined with epithelium. Individual myoepithelial cells penetrating into a dense fibrous stroma. Masson's trichrome stain. (R.I.S.H. 3004/44.) ×75.
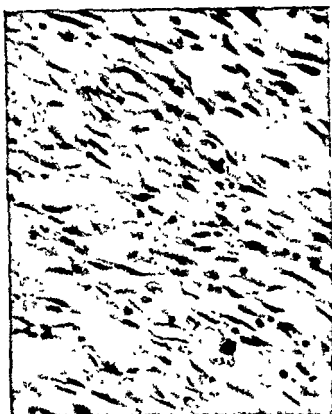
FIG. 18.—Case 4. High-power view to show the general (spindle celled) structure of the tumour. (R.I.S.H. 3044/45.) ×510.
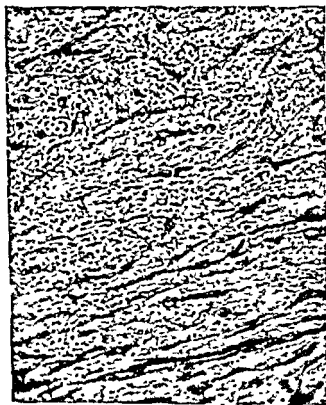
FIG. 19. Case 4. Reticulin impregnation to show epithelial type of pattern. (R.I.S.H. 3044/45.) ×110.

FIG. 20.—Case 5. Transition between myoepithelial cells and isolated cells resembling chondrocytes. (R.I.S.H. 3153/46.) ×250.

recurrence); others have retained the name Brodie's sero-cystic disease, which would appear the most appropriate. Five tumours of this kind were examined, one of which showed marked myoepithelial proliferation.

Case 6

The patient, aged 36, noticed a lump in the breast ten years prior to admission. This caused no inconvenience and did not increase greatly in size during a pregnancy; it finally formed a spherical tumour about 6 inches in diameter. Since there was no involvement of axillary lymph nodes a local mastectomy was performed. Macroscopically the tumour (R.I.S.H. 43/42) was a well encapsulated mass measuring $13 \times 12 \times 7$ cm. replacing the breast.

Microscopically the spindle-celled stroma is far more cellular than is usual in fibroadenomata and in places the structure suggests sarcomatous change. In the stroma are solid masses of epithelial cells and epithelial-lined clefts. There is a well defined myoepithelial layer composed of both vacuolate and spindle cells. As in other tumours there is a lack of sharp definition between the myoepithelium and stroma, suggesting the possibility that myoepithelial cells may have contributed to its cellularity.

The other four tumours also showed an unusually cellular stroma, but though a myoepithelial layer was always present there was no good evidence that these cells contributed to the stroma.

DISCUSSION

It is no new conception that epithelial cells may have the potentiality to develop into tissues of mesodermal appearance. The Schwann cells of the nerve sheath and the glial cells are ectodermal, yet histologically the neurilemmoma and glioma are mesodermal rather than epithelial in pattern.

The origin of muscle cells from the ectoderm is well recognised. The intrinsic muscles of the eye are ectodermal. In certain nerve tumours striated muscle may occur in the depths of the tumour remote from any mesodermal tissue (Masson and Martin, 1938). According to Masson's analysis (1938) the Wilms's embryoma of the kidney arises from ectoderm of the neural crest. These tumours not infrequently contain striated muscle. The myoepithelial cells are therefore not alone in their capacity, clearly demonstrated in animals, to form tissues of mesodermal appearance.

The human breast tumours studied are not directly comparable to those of the bitch and the importance of the myoepithelium in their morphogenesis is not so clear. Nevertheless there is some evidence that myoepithelial cells do play a part in tumour formation and it is suggested that a recognition of their possible role renders the complexity of certain tumours more easily comprehensible.

SUMMARY

1. The lesions found in mastopathy and intraduct papilloma illustrate the type of myoepithelial proliferation common in the human breast.

2. Three mixed tumours of the human breast are described and it is suggested that myoepithelial proliferation may be responsible in part for the complexity of their structure.

3. A malignant breast tumour and a carcinomatous nodule from another case, both with a possible origin from the myoepithelium, are described.

4. Of five specimens of Brodie's sero-cystic disease of the breast examined, one showed marked myoepithelial proliferation.

REFERENCES

ABRAMOW, S. S.	1901.	*Cbl. allg. Path.*, xii, 926.
BRODIE, B. C.	1846.	Lectures illustrative of various subjects in pathology and surgery, *London*, p. 137.
DURANTE, G., AND ROULLAND, H.	1921.	*Gynécologie*, xx, 389.
GAUDIER, GRANDCLAUDE AND LAMBERT, M.	1931.	*Ann. d'Anat. path.*, viii, 68.
GÜNTHER, ROSEMARIE	1937.	*Arch. path. Anat.*, ccc, 449.
HAMPERL, H.	1931.	*Z. mikr. anat. Forsch.*, xxvii, 1.
„	1939-40.	*Arch. path. Anat.*, cccv, 171.
HERTZLER, A. E., AND KOENEKE, IRENE A.	1933.	Surgical pathology of the mammary gland,. *Philadelphia* and *London*, p. 144.
VON KÖLLIKER, A.	1848-49.	*Z. für Wissensch. Zool.*, i, 48.
KUZMA, J. F.	1943.	*Amer. J. Path.*, xix, 473.
MASSON, P.	1938.	*Amer. J. Cancer*, xxxiii, 1.
MASSON, P., AND MARTIN, J. F.	1938.	*Bull. Assoc. franç. Cancer*, xxvii, 751.
MELNICK, P. J.	1932.	*Arch. Path.*, xiv, 794.
PEYRON, A.	1924.	*Bull. Assoc. franç. Cancer*, xiii, 349.
PEYRON, A., CORSY, F., SURMONT, J.	1926.	*Ibid.*, xv, 21.
PEYRON, A., AND MONTPELLIER, J.	1932.	Encyclopédie Medico-Chirurgicale, *Paris*, vol. vi, p. 907.
ROTTINO, A., AND WILLSON, KATHLEEN	1945.	*Arch. Surg.*, l, 184.
SHELDON, W. H.	1941.	*Arch. Path.*, xxxi, 326.
„	1943.	*Ibid.*, xxxv, 1.
STEIN, R. J.	1942.	*Ibid.*, xxxiii, 72.
STRONG, L. W.	1913.	*Amer. J. Obst.*, lxviii, 53.
TUDHOPE, G. R.	1939.	This *Journal*, xlviii, 499.

DEVELOPMENT OF RESISTANCE OF
SHIGELLA SHIGÆ TO GLYCINE

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GORDON and Gordon (1943) showed that the growth of some organisms on media containing sublethal concentrations of glycine was associated with cultural and morphological changes. The colonies were sticky and difficult to emulsify, and the organisms appeared elongated and markedly swollen, vibrios especially often appearing as spherical and ovoid bodies. It seemed of interest to determine whether these phenomena were associated with other changes.

Shigella shigæ (Wynne) from the National Collection of Type Cultures, which was used throughout, was inoculated on nutrient agar containing 0·25, 0·5, 1·0, 1·25 and 1·5 per cent. glycine (Analar B.D.H.). After 48 hours' incubation, there was no growth on the media containing 1·25 and 1·5 per cent. glycine and very poor growth on the agar with 1·0 per cent. glycine. This growth, which exhibited the stickiness and morphological changes produced by glycine, was repeatedly subcultured on agar containing 1·0 per cent. of glycine. The growth improved with each subculture, indicating that the organism was adapting itself to the abnormal environment. Investigations in fluid media showed that this adaptation could be extended so that growth resulted in concentrations of glycine which as a rule completely inhibited normal cultures.

Inhibition of growth of Sh. shigæ *by glycine*

One per cent. peptone broth was prepared with various concentrations of glycine, adjusted to pH 7·6, tubed in 5 ml. lots, and autoclaved. Four tubes of broth with 0·5, 1·0, 1·5 and 2·0 per cent. glycine and a control tube of broth without glycine were each inoculated with one loopful of a heavy suspension of *Sh. shigæ* in broth from an agar slope culture. After 48 hours' incubation the control broth showed marked turbidity, the 0·5 per cent. glycine broth moderate turbidity and the 1·0 per cent. glycine broth slight turbidity, but the 1·5 and 2·0 per cent. glycine broths appeared clear. Subcultures after 24 hours' incubation showed (table) heavy growths from the control broth and 0·5 per cent. glycine broth, moderate growth from the 1·0 per cent. glycine broth

and very slight growth from the 1·5 per cent. glycine broth, from which a subculture was sticky and showed marked elongation of the

TABLE

The increase of resistance against glycine acquired by Sh. shigæ *after repeated subculture in glycine broth*

Subculture in glycine broth		Resistance acquired against glycine				
Concentration of glycine (per cent.)	No. of subcultures	Concentration of glycine in broth (per cent.)				
		1·5	2·0	2·5	3·0	3·5
0·5	1-9	+	−	−	−	−
	10-20	+	+	−	−	−
1·0	1-3	+	−	−	−	−
	4-7	+	+	−	−	−
	8-19	+	+	+	−	−
	20	+	+	+	+	−
1·5	1-2	+	+	+	−	−
	3-20	+	+	+	+	−
0 (control)	1-20	+	−	−	−	−

+ = growth on subculture.
− = no growth on subculture.

bacilli. Growth was completely inhibited in 2·0 per cent. glycine broth. Gordon and McLeod (1926) and Gordon and Gordon (1943) found a similar inhibitory effect on the growth of organisms by glycine.

Development of resistance of Sh. shigæ *to high concentrations of glycine*

The cultures in 0·5, 1·0 and 1·5 per cent. glycine broth from the previous experiment were each repeatedly subcultured at 3-day intervals in broth containing the same concentrations of glycine with three loopfuls as inoculum. After 3 days' incubation each subculture was further subcultured to a series of broths containing 0·5, 1·0, 1·5, 2·0, 2·5, 3·0, 3·5 and 4·0 per cent. glycine to detect any variation in the maximum concentration of glycine permitting growth. It was found (table I) that :—

(1) After the tenth successive subculture in 0·5 per cent. glycine broth the organism was able to grow in broth containing 2·0 per cent. glycine, but even after ten further subcultures its growth was completely inhibited in 2·5 per cent. glycine broth.

(2) After the fourth successive subculture in 1·0 per cent. glycine broth the organism was able to grow in 2·0 per cent. glycine broth and after the eighth successive subculture in 2·5 per cent. glycine broth. After the twentieth successive subculture growth occurred

in 3·0 per cent. glycine broth but was completely inhibited in 3·5 per cent. glycine broth.

(3) After being grown only once in 1·5 per cent. glycine broth the organism was capable of growth in broth containing 2·5 per cent. of glycine. After the third successive subculture growth was possible in 3·0 per cent. glycine broth but not in 3·5 per cent. glycine broth. Even after 20 subcultures in 1·5 per cent. glycine broth there was no development of resistance to more than 3·0 per cent. glycine.

A control series of 20 successive subcultures at 3-day intervals in broth without added glycine showed no evidence of increased tolerance of glycine when investigated in concentrations of more than 1·5 per cent.

Repeated growth in certain concentrations of glycine normally permitting growth therefore results in the development of a capacity to grow in concentrations of glycine previously completely inhibitory, *i.e.* up to 3·0 per cent. in contrast with the normal limit of 1·5 per cent. Further investigations showed that by gradual adaptation growth could be obtained in still higher concentrations of glycine. At no stage, however, was it found possible for *Sh. shigæ* to adapt itself to increments of more than 1·0 per cent. of glycine at a time. The highest concentration in which the organism was capable of growth was ascertained by successive subculture to broths containing glycine in concentrations increasing by stages of 0·5 per cent. These were carried out at 3-day intervals starting with a primary culture in 1·5 per cent. glycine broth, 3 loopfuls being used as the inoculum in all cases. It was thus possible to obtain growth, as shown by subculture, in a concentration of glycine as high as 7·5 per cent., *i.e.* about five times the limit normally permitting growth. Some difficulty was at first experienced in obtaining growth beyond 5·0 per cent. glycine, but after several subcultures in broth containing this concentration of glycine, growth in the higher concentrations was more readily obtained. The density of growth diminished with increase of glycine and in 7·0 per cent. and 7·5 per cent. glycine there was only very slight turbidity.

Effect of repeated subculture in normal media on the acquired resistance of Sh. shigæ

The question arose whether this acquired resistance might be modified by subculturing back to normal media. Four strains of the organism were used—three obtained after 20 subcultures in (1) 0·5 per cent. glycine broth (resistant to 2·0 per cent. glycine), (2) 1·0 per cent. glycine broth (resistant to 3·0 per cent. glycine), (3) 1·5 per cent. glycine broth (resistant to 3·0 per cent. glycine), and the fourth (4) grown in 6·0 per cent. glycine broth after five subcultures in 5·5 per cent. glycine broth. All were repeatedly subcultured at 2-day intervals in normal broth and each successive

broth culture was examined for its glycine resistance by subculture into a series of broths containing increasing concentrations of glycine. After ten successive subcultures in broth, all the strains retained the original resistance, *i.e.* to 2·0, 3·0 or 6·0 per cent. glycine according to the initial resistance. The strains were again examined for their glycine resistance after five further broth subcultures made at weekly instead of 2-day intervals. The strain grown in 0·5 per cent. glycine broth lost its acquired resistance and was incapable of growth in 2·0 per cent. glycine broth. The strains grown in 1·0 and 1·5 per cent. glycine broth lost the capacity to grow in 3·0 per cent. but still grew readily in 2·5 per cent. glycine broth. The strain previously capable of growth in 6·0 per cent. glycine broth showed marked diminution in its resistance and was completely inhibited by the addition of 4·5 per cent. of glycine to broth, though it still grew in 4·0 per cent. glycine broth. After five further weekly subcultures in broth the strains grown in 1·0 and 1·5 per cent. glycine broth still maintained their resistance to 2·5 per cent. glycine and the resistance of the strain grown in 6·0 per cent. glycine broth was reduced so that it now grew only in 3·5 per cent. glycine broth.

Sugar fermentations, catalase production and agglutination properties of Sh. shigæ *grown in abnormal concentrations of glycine*

Sugar fermentations. Subcultures from all concentrations of the glycine broths used gave the same sugar fermentations as the original strain. The development of resistance to glycine therefore involved no change in this property.

Catalase production. McLeod and Gordon (1923) showed that pneumococci, streptococci and *Sh. shigæ*, in contrast with most other aerobes, produced no catalase. At all stages of this investigation subcultures were made from the glycine broth cultures to heated blood agar and examined for the presence of catalase by dropping a 1·0 per cent. solution of hydrogen peroxide on to the growth. Catalase was invariably absent.

Agglutination properties. The subcultures from glycine broth on heated blood agar were all tested by slide agglutination. Agglutination was always observed, but with the subcultures from broth containing 3·0 per cent. or more of glycine, the time for complete agglutination was markedly prolonged, being delayed up to 10 minutes for those from the highest concentrations of glycine. This delay in agglutination was reduced after the organisms had been repeatedly grown in normal broth, but in some cases the agglutination never returned to normal. The question whether the stickiness of the growth interfered with the normal agglutination would have to be explored before this delay in agglutination time can be related to any modification of the antigenic structure.

Growth of a glycine-resistant strain of Sh. shigæ *in broth containing added quantities of alanine*

The tolerance of glycine-resistant organisms for other amino acids appeared to be relevant and that for alanine, the nearest related member of the group, was investigated.

Two series of broths were prepared, each containing 0, 1·0, 2·0, 3·0, 4·0, 5·0 and 6·0 per cent. of *dl*-alanine (B.D.H.). One series was inoculated with a 4-day broth culture of normal *Sh. shigæ*, the other with a 4-day culture of the same organism grown in 6·0 per cent. glycine broth. After three days' incubation subculture showed that the normal organism grew in 3·0 per cent. and failed to grow in 4·0 per cent. alanine broth. In striking contrast the organism capable of growth in 6·0 per cent. glycine broth was also able to grow in broth containing 6·0 per cent. alanine. Thus *Sh. shigæ*, in developing resistance to abnormal concentrations of glycine, becomes resistant also to abnormal concentrations of alanine. The effect on other amino acids was not investigated.

The glycine content of media before and after growth of Sh. shigæ

In a consideration of the mechanism enabling *Sh. shigæ* to grow in apparently abnormal concentrations of glycine, it was essential to exclude breakdown of the amino acid by the organism and reduction of its concentration to the normal limits permitting growth. Four lots of 25 ml. of media were prepared, two of normal broth and two of 5·0 per cent. glycine broth. One of the normal broths was inoculated with five loopfuls of a 4-day broth culture of *Sh. shigæ* and one of the 5·0 per cent. glycine broths with the same amount of a 4-day 6·0 per cent. glycine broth culture. One normal broth and one 5·0 per cent. glycine broth remained uninoculated. All four tubes of media were incubated at 37° C. for one week. A good turbid growth was obtained in the normal broth and a fair growth in the 5·0 per cent. glycine broth. Both cultures were sterilised by heating at 65° C. for 30 minutes and the uninoculated media were also subjected to this treatment. Since all possible modes of bacterial breakdown of glycine involve the production of either ammonia or methylamine, the total amount of amino nitrogen in each of the four media was determined by formol titration and the amount of ammonia nitrogen by the Conway micro-diffusion method. It was found that there was no decrease in the total amino nitrogen in either of the inoculated media as compared with the corresponding un-inoculated tubes. There had therefore been no loss of volatile nitrogen compounds during the process of sterilisation and also no appreciable amount of nitrogen built up into the protein of the organisms. Slight increases in the ammonia nitrogen were found in both the inoculated media as compared with the corresponding uninoculated tubes, but

the increase in the glycine broth culture was less than that in the normal broth culture. The slight difference could quite well be accounted for by the more vigorous growth in normal broth. It is evident, therefore, that the ability of *Sh. shigæ* to grow in high concentrations of glycine is not associated with breakdown of glycine and reduction of its concentration to the limits normally permitting growth.

DISCUSSION

The growth of *Sh. shigæ* is completely inhibited by the addition of 2·0 per cent. glycine to broth, but by gradual adaptation of the organism to increasing concentrations growth becomes possible in broth containing as much as 7·5 per cent. glycine. The ability of an organism to develop resistance against an inhibitory agent has long been recognised since Ehrlich (1907) demonstrated that trypanosomes could become resistant to dyes. The recent introduction of the sulphonamides, penicillin and streptomycin still further stimulated interest in the subject when it soon became evident that " fastness " of bacteria to these chemotherapeutic agents was of increasing practical importance. The resistance acquired by *Sh. shigæ* to abnormal concentrations of glycine presents features similar to that shown by organisms resistant to the antibiotics and sulphonamides. Thus resistance to glycine is usually a gradually developing process, for though growth of the organism is ultimately possible in 5 times the maximum concentration of glycine which normally permits growth (7·5 compared to 1·5 per cent.), adaptation is able to proceed only by increments of not more than 1·0 per cent. glycine at each stage. Kirby and Rantz (1943, p. 38), in their studies of sulphonamide resistance, found that " the degree of resistance developed is greatly influenced by the concentration of drug employed " and " that unless the drug is present in sufficient concentration to inhibit the growth of the organisms, very little resistance is developed ". Similarly repeated growth of *Sh. shigæ* in a mildly inhibitory concentration of 0·5 per cent. glycine resulted in the development of resistance to only 2·0 per cent. glycine ; and even after repeated growth in a sublethal concentration of 1·5 per cent. glycine, resistance reached only 3·0 per cent. glycine compared with a possible maximum of 7·5 per cent. attained by gradually training the organism to increasing concentrations. Again, it is recognised that when resistance against sulphonamide is well established, it is retained almost indefinitely (Henry, 1944), and similarly persistent resistance to abnormal concentrations of glycine is shown by *Sh. shigæ* after repeated subculture of a resistant strain in normal media free from added glycine.

It is worthy of note that this acquisition of resistance to glycine is not accompanied by any changes in other characteristics of the organism such as the sugar fermentations and the non-production of

catalase. Agglutination by antisera appears to be delayed, but this may be related to the sticky nature of the growth associated with high concentrations of glycine.

The mechanism of the development of glycine resistance by *Sh. shigæ* is not known, but there is no evidence that it is accompanied by any abnormal destruction of glycine by the organism.

SUMMARY

1. *Sh. shigæ* grows poorly in broth containing 1·5 per cent. glycine and is completely inhibited in 2·0 per cent. glycine broth.

2. Repeated subculture of the organism in broth containing 0·5, 1·0 and 1·5 per cent. glycine enables it to grow in broth containing 2·0, 3·0 and 3·0 per cent. glycine respectively.

3. Subculture of the organism to broth containing successive increments of 0·5 per cent. glycine enables it to grow in 7·5 per cent. glycine broth.

4. Repeated subculture of the highly resistant strains in normal broth resulted in some loss of resistance, but it persisted to previously inhibitory concentrations of glycine.

5. A glycine-resistant strain of *Sh. shigæ* showed similar resistance to alanine.

6. There was no evidence that glycine destruction by the organism reduced the concentration of the amino acid to limits normally permitting growth.

REFERENCES

EHRLICH, P.	. . . . . .	1907.	*Berl. klin. Wschr.*, xliv, 233, 280, 310 and 341.
GORDON, J., AND GORDON, M.	.	1943.	This *Journal*, lv, 63.
GORDON, J., AND McLEOD, J. W.	1926.	This *Journal*, xxix, 13.
HENRY, R. J.	. . . . . .	1944.	The mode of action of sulfonamides, Publication of Josiah Macy Jr. Foundation, Review series, vol II, no. 1, *New York*.
KIRBY, W. M. M., AND RANTZ, L. A.	1943.	*J. Exp. Med.*, lxxvii, 29.
McLEOD, J. W., AND GORDON, J.	1923.	This *Journal*, xxvi, 326.

THE ESSENTIAL LESION OF PNEUMOKONIOSIS IN WELSH COAL WORKERS

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(PLATES LXV-LXVIII)

In their study of pneumokoniosis in South Wales coal workers, Belt and Ferris (1942) described the primary lesion as a " dust-reticulation ", consisting of a diffuse pigmentation of the framework of the lung associated with a more or less uniform increase of reticulum fibres. It is true that they also cited a condition of " nodular reticulation ", but this was regarded as a later manifestation of the disease. They therefore represented the first change in the pneumokoniosis of coal workers as being essentially diffuse, but it must be pointed out that they examined only relatively advanced cases, the minimum period of exposure being 18 years. Their view is at variance with the earlier report of Cummins and Sladden (1930), who examined a group of South Wales coal workers, most of whom were anthracite miners, and it has since been opposed, also from South Wales, by Gough (1944) and Williams (1944). All these authors hold that the earliest lesion is characteristically focal in distribution. The pneumokoniosis of coal workers in other countries has also been regarded as a diffuse condition, for instance in Germany (Husten, 1931), England (Stewart, 1934), America (Gardner, 1933a, 1935; Miller, 1935; Walsh, 1938) and Australia (Badham and Taylor, 1936, 1939). A parallel difference of opinion with regard to the primary lesion occurred in the study of classical silicosis. Gardner (1933a) in America described a linear perilymphatic fibrosis as the initial effect, whereas the South African workers (Strachan and Simson, 1930; Simson, Strachan and Irvine, 1931; Simson, 1935; Simson and Strachan, 1935), observing the disease in gold miners from the Witwatersrand, concluded that the earliest change was sharply localised to the divisions of the respiratory bronchioles. A study of the lungs of South Wales coal workers leads me to the conclusion that their pneumokoniosis is primarily and essentially focal, and in reaching this conclusion I have had the advantage of examining much earlier cases than previous investigators.

Materials and methods

The lungs of 85 Welsh coal workers, most of whom were employed in steam-coal (*i.e.* carbonaceous- and semibituminous-coal) mines, but including 7 anthracite miners, 2 bituminous-coal miners and 5 steam-coal trimmers, have been examined. The average duration of their working life was approximately 33 years, although individually it ranged from 1 to 60 years. The miners comprised hard headers, borers, rippers, firemen, shotsmen, fillers, labourers, packers, hauliers, riders, repairers and surface workers, and, so far as could be ascertained, many had been engaged in more than one of these occupations and at more than one colliery. The commonest causes of death were cardiovascular disease—sometimes related to the lung condition, sometimes not—and pulmonary tuberculosis.

In order to obtain as true a representation as possible of the anatomical relationships of the focal lesion, the lungs were distended at a pressure of less than 100 mm. Hg. to approximately natural size with formol-saline via the main bronchi. The latter were then clamped and the lungs immersed in the same fixative for at least 24 hours. No evidence could be found that this caused any damage. Portions of material were taken from the upper lobe of the left lung whenever possible and embedded in paraffin wax *in vacuo*. Large histological sections, serial in certain instances, were employed to demonstrate the general features of the lesion, and small ones, always serial, for more detailed studies. Sections were stained with hæmatoxylin and eosin and by the silver impregnation method of Laidlaw.

Morbid anatomy

On section the lungs showed an average of 2-5 macules of blackened parenchyma, 1-4 mm. in diameter, in each secondary lobule. In certain cases they tended to diminish in size and number towards the base, but otherwise their distribution was more or less symmetrical. The smaller lesions were rounded or oval, whilst the larger had an irregular or stellate outline. Around the latter were enlarged air spaces, each of which in the more advanced lesions measured as much as 4 mm. in diameter. Occasionally one or two specially large spaces occurred to the side of the pigmented area. Some lungs showed in addition small fusiform collections of pigment in the septa, particularly at their junctions, but these were quite distinct from the focal accumulations in the vesicular tissue and were not as a rule accompanied by emphysema. In the pleura soft black plaques could usually be seen, often connected by black lines demarcating the secondary lung lobules.

Histology

In order to determine the form of the initial lesion special attention was paid to such cases as that of a boy of 15 who had spent but one year at the coal face and two young men who had had only four and five years respectively underground. In these the dust seems to be collected mainly at the divisions of the respiratory bronchioles (fig. 1), being located in the connective tissues which surround them together with their accompanying arterioles (fig. 2), and which normally contain small aggregations of lymphoid tissue (Simson and Strachan, 1931;

FIG 1—Early dust lesion at the division of a respiratory bronchiole   ×10

FIG 2—Early lesion in transverse section, showing interstitial collection of dust around three branches of a respiratory bronchiole and their accompanying arterioles ×25.

FIG 3—From the same lesion as fig 2 Interstitial accumulation of dust cells Reticulum impregnation   ×300
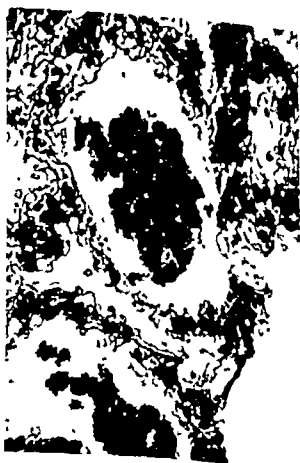
FIG 4—Edge of an early lesion Accumulation of dust phagocytes in the alveoli and alveolar walls Reticulum impregnation hæmalum and eosin   ×300

FIG 5 —Larger dust lesion in the formative phase No focal emphysema ×25

FIG 6 —From the same lesion as fig 5 Reticulum impregnation ×300
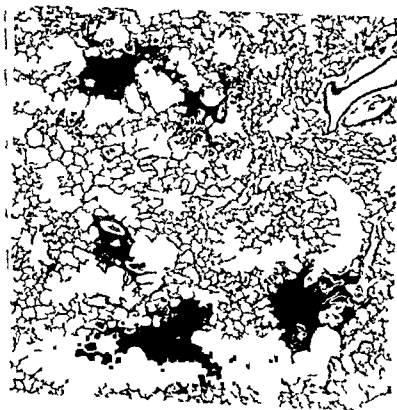
FIG 7 —Formative and early retrogressive changes in the same lesions Focal emphysema commencing ×10
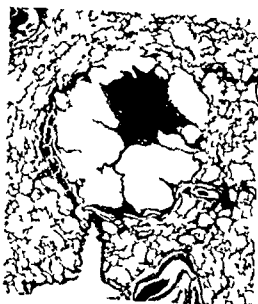
FIG 8 —Retrogressive dust lesion from a coal miner Focal emphysema now present together with peripheral collection of dust ×10

Miller, 1937). There, pigmented foci $\frac{1}{2}$-2 mm. in diameter are formed, in which the dust appears to be partly contained in phagocytes and partly free. As a rule the dust is so thickly concentrated as to obscure the tissues, although silver impregnation reveals delicate reticular fibres running through the foci (fig. 3). Frequently dust is also present outside the interstitial tissues, adjacent alveoli being occupied by masses of dust-bearing cells, while their intervening walls, though they show no actual increase of connective tissue, are thickened by infiltration with these phagocytes (fig. 4). Sometimes tiny accumulations of dust also occur around venules at the periphery of the primary lobules of the lungs. It must be realised that some miners with much longer periods of exposure to dust have no more than these early lesions in their lungs.

The majority of specimens contain larger lesions than those just described, measuring 2-4 mm. in diameter, but with the same anatomical distribution. In describing them it is necessary to take into account the fact that the histological picture varies not only with their size but also with their stage of development, some being in the formative phase while others show changes indicative of retrogression. In the formative phase of their development the vesicular tissue in the affected areas is overrun with dust and dust-laden phagocytes, producing localised consolidations which tends to obscure the lung structure (fig. 5). Here and there the outlines of some alveoli can be made out, but for the most part both the alveoli and their walls are so densely packed with cells that they were fused into solid masses of blackened tissue in which the only supporting framework is a fine reticulum (fig. 6). These appearances are usually most marked towards the centre of the lesions. At the periphery the vesicular structure is more in evidence, although here also the spaces are filled with dust cells and their walls more or less infiltrated with them. The outlines of the lesions are usually fairly abrupt, the surrounding air spaces being mostly free from dust and at this stage practically normal in size. In the retrogressive phase, on the other hand, the lesions are still more dense and compact, the vesicular structure eventually being obliterated throughout, and there is now a distinct formation of collagenous connective tissue lying fairly evenly amongst the dust. The most important feature of these larger lesions, however, is the emphysema localised to the vesicles immediately around them. The emphysematous spaces are partially bounded by the macules and by tapering areas of consolidation which extend from their margins, but elsewhere the walls of these spaces are composed of stretched and thinned alveolar septa. In this way the lesions come to have a stellate outline (figs. 7, 8 and 9). Such a formation suggests that in the production of the emphysema shrinkage of the focal lesions has played a part. The degree of emphysema varies widely in different cases and does not necessarily correspond with the size of the nodule. In some cases there are small consolidations, or small

accumulations of dust cells in the alveoli and alveolar walls, lying immediately outside the emphysematous area (figs. 8 and 9). It must be recognised that generalised vesicular emphysema occurs independently in certain of these lungs, but as a rule it does not obscure the focal lesion. On the other hand the focal emphysema is occasionally so extensive as to leave but little of the parenchyma unaffected.

In a number of the more advanced cases dust also accumulates in places other than the terminations of the respiratory bronchioles, the commonest being their origins: sometimes, especially if they are short, these bronchioles are completely surrounded by cuffs of pigment. Small amounts of dust are also found occasionally at the distal ends of the alveolar ducts and many specimens show a little pigment along segments of the larger air passages and their blood vessels. In a few subjects, particularly those who have recently been working, scattered dust cells are seen lying free in the alveoli throughout the lungs.

Several lungs exhibit lesions which are so extensive and close-set as to produce a condition which might be described as diffuse (fig. 10). Even in them, however, unaffected areas are readily recognisable which proves at least that the condition is not altogether diffuse. The fibrosis is distinctly patchy, being most plentiful where there is most dust, and, although in these advanced cases the emphysema is not so clearly referable to the consolidated areas as when the dust foci are quite discrete, it also shows a patchy distribution. Thus the whole picture strongly suggests that the focal lesions, having increased in size, have become more or less confluent.

Discussion

To understand the peculiar distribution of dust within the lung and the form of the lesions which develop, it is necessary to consider the manner in which the lung deals with inhaled particles. Air-borne dust of respirable size is generally believed to be deposited uniformly in the alveoli and to be rapidly ingested by phagocytes. Many of these cells are no doubt expectorated but some find their way into the interstitial tissues. Gardner (1940) held, largely on the basis of his experimental observations, that phagocytes take the dust along the air spaces into the nearest lymph vessels, through which the dust is then transported to the hilar glands, where it comes to rest. He considered that the accumulation of dust steadily extends towards the periphery of the lung and involves both the lymphoid depots and the lymph vessels, but only when no more dust can be accommodated in the hilar glands. Cummins and Sladden evidently believed that phagocytes, having carried the dust straight into the alveolar walls, migrate along their tissue spaces into the perivascular and peribronchial lymphatics and so to the hilar glands, but how the focal lesions arise was not discussed by them. My observations suggest
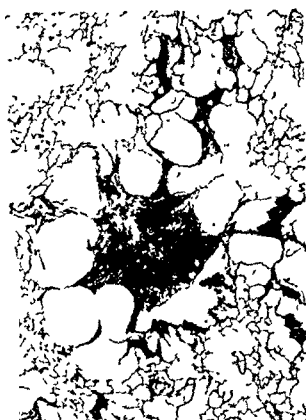
FIG. 9 —Similar lesion to fig 8 but from a coal trimmer   ×10

FIG. 11 —Focal collection of dust in a man who had always been a coke worker.   ×25.
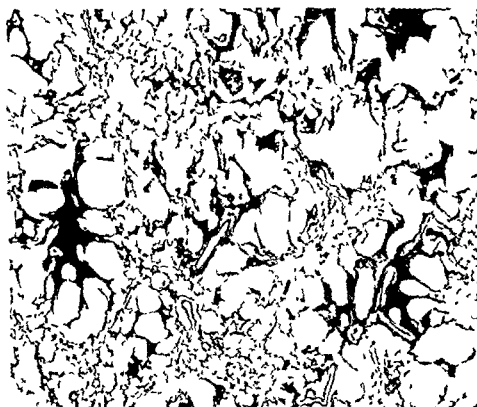
FIG 10 —Advanced dust disease showing extensive focal lesions with focal emphysema; becoming confluent   ×5

that as a rule the phagocytes do not immediately pass into the alveolar walls but migrate by way of the air spaces to the vicinity of the lymphoid aggregations which are situated at the divisions of the respiratory bronchioles, where they then pass into the interstitial tissue and enter the lymphatic system. In pneumokoniosis more dust-laden phagocytes reach these depots than can be disposed of, so that they become choked with cells. An accumulation of cells then occurs in the vesicles around these points and consequently localised areas of lung parenchyma are crowded with phagocytes, giving an appearance of consolidation by accumulation of masses of dust, and as more cells arrive the process gradually covers wider areas of vesicular tissue. The next stage seems to be the formation throughout these areas of a fine network of reticular fibres, which in time tend to become collagenous. In this way the focal accumulations of dust undergo a slow organisation, and by this means the enclosed cells are prevented from wandering further. Cicatrisation naturally follows and with it shrinkage and condensation of the lesions.

There have been many references in the literature to generalised or bullous emphysema but few to the localised form in connection with coal workers' pneumokoniosis. Belt and Ferris noted that emphysema often coexisted with dust lesions but made no more than passing reference to it. Cooke (1932) and Gardner (1933b, 1940) mentioned enlarged air spaces in relation to coal-dust lesions, although neither of them seemed to attach any importance to the emphysema. As long ago as 1834, however, T. W. Jones, in a report to Dr G. Hamilton (1834, p. 299), emphasised, in the case of a Scottish collier whose lungs he had examined, that the air cells were most dilated where the black matter was most abundant. Badham and Taylor (1936, 1939) also stressed the fact that emphysema, when present in the lungs of coal miners in New South Wales, is particularly evident around the dust lesions, considering that the dust fibrosis causes the emphysema and that the latter is the factor underlying the clinical disability. Similarly Gough (1940), reporting on 12 steam-coal trimmers from Cardiff, referred to the characteristic emphysematous spaces around the nodular lesions. He further pointed out (Gough, 1944), as did Williams (1944), that in Welsh coal workers as a whole emphysema in and around the focal collections of dust is a common finding. In the series of lungs which I have examined the emphysema is so strictly localised to the vicinity of the focal consolidations as to suggest that there is a " cause and effect " relationship. Early lesions do not show the focal emphysema, whilst the more advanced ones do, so that dust collection appears to be the primary change. The mechanism by which the emphysema is induced still remains a matter of inference. As, however, the dust lesions are essentially areas of consolidation which are unable to expand on inspiration, the adjacent alveoli must become enlarged and the alveolar walls stretched, and

this effect will be augmented by fibrous contraction in the lesions themselves. The emphysema is thus vicarious in type. It may well be that it is this focal emphysema which, by leading to defective hæmo-respiratory exchange, is the main factor underlying the dyspnœa so frequently present in the earlier phases of the disease.

The composition and the amount of dust inhaled in the various occupations concerned with coal mining differ, sometimes considerably, yet the same focal type of lesion occurs in miners irrespective of whether they were employed in anthracite, carbonaceous-coal, semi-bituminous-coal or bituminous-coal mines and, with few exceptions, it seems to make little difference whether they had worked mainly at the coal face, in the associated strata of rock or on the roadways. At the docks, coal trimmers handle coal only and are thus exposed to relatively pure coal dust, but the lesions of steam-coal trimmers show no fundamental differences from those of the miners (compare figs. 8 and 9). Furthermore, I have found comparable changes in coke workers (figs. 11 and 12) and in iron workers, whilst the lesions in the lungs of town dwellers (figs. 13 and 14) are indistinguishable from the early lesions of coal miners. These considerations suggest that the development of the lesions in coal workers may be determined to a significant extent by the mechanical accumulation of a sufficient amount of dust, irrespective of its nature. This view is rendered the more likely since the evidence for the alternative silica hypothesis is not completely satisfactory (King and Nagelschmidt, 1945; King, 1945). di Biasi (1939) has also suggested that coal-dust particles influence the lung mechanically. The amount of dust retained in the lung and consequently the degree of disability attributable to the dust cannot be correlated with known duration of work in the industry nor, apparently, with the particular occupation in which the men were chiefly engaged. Under comparable circumstances there are great variations in the amount of dust which different persons accumulate in their lungs. These variations may depend upon the efficiency of the mechanisms for dust elimination such as nasal filtration, bronchial movement and secretion, ciliary action and phagocytosis, all of which may vary either naturally or as a result of disease.

The simple silicotic nodule (Simson, 1935; Belt, 1939) differs from the focal lesion of coal workers in several respects. Pigmentation is considerably less and is unevenly distributed, whereas the amount of collagenous connective tissue is much greater and has a characteristically whorled or concentric disposition (fig. 15). According to Simson and Strachan (1935) the fibrous overgrowth is evident at an early stage in the development of silicosis, apparently extending from the centre of the early silicotic nodule and undergoing gradual hyalinisation, but in coal workers the fibrosis is never obvious and occurs uniformly and sometimes radially throughout the focal lesion. Perhaps the most important difference is the relatively minor degree

WELSH COAL WORKERS' PNEUMOKONIOSIS

FIG 12.—From the same lesion as
fig. 11.   Reticulum impregnation.
×300

FIG. 13.—Normal lung   Focal aggregation
of dust at the division of a respiratory
bronchiole.   ×25.

FIG 14 — Similar lesion to fig 13   Reticu
lum impregnation   ×300.

FIG. 15 —Well developed simple silicotic
nodule for comparison   Note almost
complete absence of focal emphysema
×10

of focal emphysema which occurs around the silicotic nodule. These points of distinction are sufficiently great as to suggest that the pathogenesis of the two lesions is fundamentally different, but exactly how it differs it is still too soon to say.

Conclusions

In the coal workers of South Wales the primary lesion of pneumokoniosis is a focal accumulation of dust, which is situated at the division of the respiratory bronchiole. The lymphoid tissue at this point is the main factor in determining the localisation of coal dust within the lung. No matter how extensive the disease, the dust lesion remains essentially focal in distribution. In the more advanced lesion a small amount of fibrosis does occur but its most characteristic feature is emphysema strictly localised to the adjacent vesicles. It is suggested that the focal dust lesions cause the focal emphysema mechanically and that the latter is mainly responsible for the respiratory disability of the uncomplicated disease. Since comparable lesions occur in men with different industrial histories as well as in people with no occupational dust hazard whatever, it is also suggested that the mechanical accumulation of dust, irrespective of its nature, plays a significant part in the pathogenesis of the dust lesions.

REFERENCES

BADHAM, C., AND TAYLOR, H. B. 1936. Studies in industrial hygiene, no. 19. (Extract from Ann. Rep., Dept. of Publ. Hlth., New South Wales).

,, ,, ,, ,, 1939. *Ibid.*, no. 20. (Extract from Ann. Rep., Dept. of Publ. Hlth., New South Wales).

BELT, T. H. . . . . 1939. This *Journal*, xlix, 39.

BELT, T. H., AND FERRIS, A. A. . 1942. Medical Research Council, Spec. Rep. Ser., no. 243, *London*, p. 203.

DI BIASI, W. . . . . . . 1939. *Dtsch. med. Wschr.*, lxv, 369.

COOKE, W. E. . . . . . . 1932. *Practitioner*, cxxix, 483.

CUMMINS, S. L., AND SLADDEN, A. F. 1930. This *Journal*, xxxiii, 1095.

GARDNER, L. U. . . . . . 1933a. *J. Amer. Med. Assoc.*, ci, 594.

,, ... 1933b. *In* Brundage, D. K., and Frasier, Elizabeth S., U.S. Publ. Hlth. Bull., no. 208, *Washington*, p. 16.

,, ... 1935. *Internat. Clin.*, series 45, ii, 16.

,, ... 1940. *J. Amer. Med. Assoc.*, cxiv, 535.

GOUGH, J. . . . . 1940. This *Journal*, li, 277.

GOUGH, J. . . . . . . 1944. Report of the Advisory Committee on Treatment and Rehabilitation of Miners in the Wales Region suffering from Pneumokoniosis, *London*, p. 17.

HAMILTON, G. . . . . . . 1834. *Edinb. Med. and Surg. J.*, xlii, 292.

HUSTEN, K. . . . . 1931. *Klin. Wschr.*, x, 506.

KING, E. J. . . . . 1945. Medical Research Council, Spec. Rep. Ser., no. 250, *London, p. 89*.

KING, E. J., AND NAGELSCHMIDT, G. 1945. Medical Research Council, Spec. Rep. Ser., no. 250, *London*, p. 18.

MILLER, J. W. . . . . . . 1935. U.S. Publ. Hlth. Bull., no. 221, *Washington*, p. 69.

MILLER, W. S. . . . . . . 1937. The lung, *Springfield, Ill.* and *Baltimore, Md.*, p. 115.

SIMSON, F. W. . . . . . . 1935. This *Journal*, xl, 37.

SIMSON, F. W., AND STRACHAN, A. S. 1931. Publications of the South African Institute for Medical Research, vol. iv, no. 26, p. 231.

" " " 1935. Publications of the South African Institute for Medical Research, vol. vi, no. 36, p. 367.

SIMSON, F. W., STRACHAN, A. S., AND IRVINE, L. G. 1931. Proceedings of the Transvaal Mine Medical Officers' Assoc., vol. x, no. 118, supplt.

STEWART, M. J. . . . . . . 1934. *Edinb. Med. J.*, xli, 226.

STRACHAN, A. S., AND SIMSON, F. W. 1930. International Conference on Silicosis, Johannesburg, I.L.O., series F, no. 13, p. 223.

WALSH, J. . . . . 1938. *Amer. Rev. Tuberc.*, xxxviii, 363.

WILLIAMS, ENID . . . . . . 1944. Report of the Advisory Committee on Treatment and Rehabilitation of Miners in the Wales Region suffering from Pneumokoniosis, *London*, p. 18.

A NEW MEDIUM FOR THE ISOLATION AND IDENTIFICATION OF *C. DIPHTHERIÆ* BASED ON THE PRODUCTION OF HYDROGEN SULPHIDE

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(PLATE LXIX)

IN most laboratories the diagnosis of diphtheria is based upon (*a*) the naked-eye appearance of the colonies and the morphology of the organisms on tellurite-blood-agar (TBA) and (*b*) the morphology on inspissated serum. This entails examination of the cultures on both media, both at the end of the first day and on the second day, if a number of positives are not to be missed. Even at the end of this period, owing to the extreme pleomorphism of some diphtheroids and the tendency of certain strains of *Corynebacterium diphtheriæ* to approximate to a diphtheroid and more uniform morphology, there may still be considerable doubt about the identity of the organisms, and further investigations may be necessary before a result can be given. Such doubts and delays lessen the value of the method, and any test which would resolve these doubts and shorten the time required would be of the greatest assistance and would much increase the usefulness of the method. The production of H_2S under the conditions described below would appear to be such a test.

EXPERIMENTAL OBSERVATIONS

During a series of experiments on the effect of certain sulphur-containing compounds on the growth of *C. diphtheriæ*, dark brown haloes were observed round the colonies of the organism on serum-agar plates containing *l*-cystine and potassium tellurite. On investigation, these haloes were found to arise from the interaction of the potassium tellurite and the H_2S produced by the bacilli from the *l*-cystine. The haloes often appeared so slowly, three or four days being sometimes required, or were so faint that they were of little value in identifying *C. diphtheriæ*, though they were never seen round colonies of the diphtheroids examined at that time.

With *Bacterium coli*, Desnuelle (1939) has shown that, previous to desulphuration, *l*-cystine is reduced to *l*-cysteine and that the

presence of hydrogen donators increases the rate at which H_2S is produced. It was thought, therefore, that the same effect might be obtained with *C. diphtheriæ*. Suitable amounts of reducing agents were added to the medium and, though all of them hastened the appearance of the haloes, sodium thiosulphate proved to be by far the most satisfactory compound for the differentiation of *C. diphtheriæ* from other organisms.

Composition of the medium

(*a*) Agar made from 1 per cent. Lemco, 1 per cent. Evans peptone, 0·5 per cent. NaCl, and 2½ per cent. agar, at *p*H 7·4. (*b*) Fresh horse serum sterilised by filtration. To 100 ml. add 10 ml. Liq. Trypsin Co. (Allen & Hanbury), mix, place in a water-bath at 37° C. for four hours and then heat at 60° C. for 30 minutes on three successive days. (*c*) Sheep blood, prepared with formalin and ether as described by Wilson (1934). (*d*) *N*/10 NaOH. (*e*) *l*-Cystine, a 0·4 per cent. solution in *N*/10 HCl, sterilised by heating in the water-bath at 60° C. for 30 minutes and stored in the refrigerator. (*f*) Potassium tellurite (B.D.H.), 1 g., dissolved in 100 ml. of boiled and cooled distilled water and stored in refrigerator. (*g*) Sodium thiosulphate (Analar), 1 g., dissolved in 40 ml. of distilled water which has been boiled and rapidly cooled. This is heated at 60° C. in the water-bath for 30 minutes and stored in the refrigerator.

To 100 ml. of (*a*) at 55° C. add, in this order, 15 ml. (*b*), 0·3 ml. (*c*), 6·0 ml. (*d*), 6·0 ml. (*e*), 3·0 ml. (*f*), 1·7 ml. (*g*). Thorough mixing during and between each addition is essential. The *N*/10 NaOH should be checked frequently against the *N*/10 HCl used to dissolve the cystine so that no change in *p*H follows the addition of these to the agar.

The medium, which should be quite transparent, has a faint orange colour which fades to straw yellow on incubation. It will not keep, but if necessary the plates may be made without the sodium thiosulphate, when they will keep indefinitely. The thiosulphate is subsequently dropped on to the surface of the plates in the proportion of 0·125 ml. per 3½ in. plate and allowed to soak in after gentle spreading. The plates should be warmed in the incubator immediately beforehand.

Appearance of the colonies of organisms which grow on the medium

C. diphtheriæ. The colonies (figs. 1 and 2) appear in 14-24 hours, though their appearance may be delayed for 36 hours, especially if only very small numbers are present. Shortly after they become visible, faint brown haloes can be detected round the colonies. These haloes are about 1 mm. wide and have sharply limited outer edges. As the colonies increase in size, the haloes increase in intensity until

CULTURE MEDIUM FOR *C DIPHTHERIÆ*

Fig 1 —Confluent 20 hour growth of *C diphtheriæ mitis*
Direct plating of throat swab   ×1¼
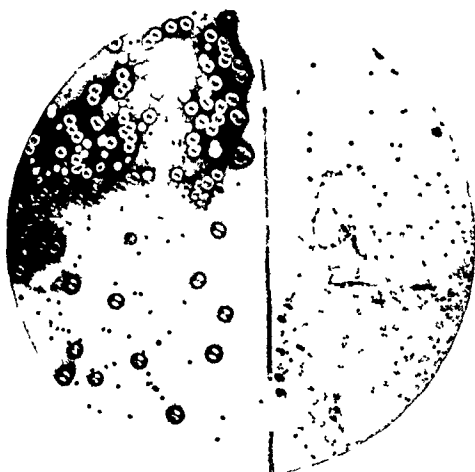
Fig 2 —The left side of the plate is a 40 hour culture of a throat swab showing
streptococci (small black colonies) diphtheroids (black, flattish colonies with no
halo) and *C diphtheriæ gravis* (large shining colonies with a black halo)   The
white ring round the colonies at the top is due to the fresh grey growth piling
up round the initial black colonies   note the lessened intensity of the discolouration
around these   The culture on the right shows only streptococci and a few
diphtheroids   Natural size

they are almost black, but they still retain their sharp edges and no diffusion takes place into the surrounding area. The colonies themselves are regular, round, domed and shining and look like small segments of highly polished black glass spheres. *Intermedius* and *mitis* strains retain this appearance indefinitely, but not *gravis* strains and the irregular strains, which rapidly revert to an alkaline *p*H in broth; with these, as incubation proceeds, the intensely black colonies become slightly greyish, the haloes become more diffuse and less marked, and new light grey growth is formed around and piled up on the original colonies (fig. 2). The time required for this transformation depends upon the speed at which the culture reverses the initial acid reaction in broth. The morphology of the organisms on the medium resembles that of a short, squat diphtheroid, though considerable pleomorphism is usually present.

Diphtheroids. These grow on the medium just as on TBA. In our experience the great majority, including those from nasal swabs which simulate *intermedius* strains, produce no discolouration of the medium; but on the second day, especially if there is confluent growth or the colonies are close together, a few show slight diffuse browning. This browning is observed only around colonies which have already attained a considerable size on the first day and is unlikely to cause confusion once the characteristic appearance of *C. diphtheriæ* has become familiar. The appearance of the colonies themselves is similar to that on TBA and ranges from white through all shades of grey to black.

Streptococci. These occasionally grow on the medium in small, flattish black colonies which attain their maximum size in 24 hours. If they grow in large numbers and only a few *C. diphtheriæ* are present, they tend to interfere with halo production because of the formation of hydrogen peroxide. A small amount of blood is therefore added to the medium to reinforce the catalase present in the colonies of *C. diphtheriæ*; in addition the blood seems to increase the inhibitory action of the potassium tellurite for streptococci.

Staphylococci. These rarely grow on the medium. Of those that do many fail to produce any discolouration, but a few simulate the appearance of diphtheroids; *i.e.* a faint diffuse browning appears after the colonies have grown to a considerable size.

Proteus. This organism may grow from certain ear swabs. If seen before they have spread the colonies are flat, greyish-black, dull and regular and are surrounded by a dirty grey zone about 3 mm. wide, quite unlike that of *C. diphtheriæ*.

Results with the medium

Tables I–III give the results so far obtained with the medium. Each colony was detected merely by its appearance and picked off to TBA and Loeffler's serum; its fermentation of dextrose, sucrose

and starch was determined and its growth on phenol-red broth noted. All were morphologically and biochemically *C. diphtheriæ*.

TABLE I

Swabs from cases of suspected clinical diphtheria

Source	TBA		New medium		Total
	Positive	Negative	Positive	Negative	
Throat swabs . .	33	380	36	377	413
Nasal swabs . .	1	23	1	23	24
Ear or vaginal swabs .	0	7	0	7	7

Of the 33 positives on TBA, one was subsequently found to be a diphtheroid ; it was negative on the new medium. Of the 4 positives on the new medium which were negative on TBA, 3 contained only a few colonies and were not from clinical cases of diphtheria. The other was a strain of *C. diphtheriæ* sensitive to small amounts of potassium tellurite when grown on TBA. Several other sensitive strains have been met with and all grow well on the new medium.

TABLE II

Swabs from contacts

Source	TBA		New medium		Total
	Positive	Negative	Positive	Negative	
Throat swabs . .	5	162	6	161	167
Nasal swabs . .	2	6	2	6	8

TABLE III

Swabs from convalescents

Source	TBA 1st day		TBA 2nd day		New medium 2nd day		Total
	Positive	Negative	Positive	Negative	Positive	Negative	
Throat swabs .	89	316	131	274	148	257	405
Nasal swabs .	13	85	13	85	14	84	98
Ear swabs .	0	5	0	5	0	5	5

There is marked discrepancy between the results of the first- and second-day TBA plates because many swabs are not received until late

in the afternoon and have thus only 14–15 hours' incubation before being examined on the following morning. With 24 hours' growth the figures would be higher, though they would still be below the second-day figures.

In addition to the results in tables I–III, 641 swabs were examined from cases in which classification was impossible owing to lack of information as to source and other particulars. Of these, 60 were positive on TBA and 93 on the new medium ; the 93 included 13 strains sensitive to tellurite on TBA.

Of the strains examined, 28 per cent. were *mitis*, 37 per cent. *gravis*, 22 per cent. *intermedius* and 13 per cent. irregular.

Discussion

The production of H_2S by *C. diphtheriæ* was first described by Petri and Maassen (1893) testing with lead acetate papers in broth containing varying amounts of Witte's peptone. Bürger (1914) described its production from cystine on lead acetate agar containing sheep serum and noted haloes round the colonies in 48 hours. These observations were not mentioned in the M.R.C. monograph on diphtheria (Andrewes *et al.*, 1923) or in the System of bacteriology (Bulloch *et al.*, 1930) and we were unaware of them until after the mechanism of halo production had been worked out. Potassium tellurite does not appear to have been used previously as an indicator of H_2S production.

Although *C. diphtheriæ* is a very vigorous producer of H_2S, especially for a non-intestinal aerobe, this in itself does not account for the highly characteristic appearance of the colonies on the medium. The key to this would seem to be found in the changes in the halo and colony appearance in strains which rapidly reverse the initial acid reaction in broth. From these changes it would appear that it is this acid reaction which causes the retention of the H_2S around the colonies and in this connection there are two points worthy of note. One is that in the quantitative estimation of small amounts of H_2S, the solution of zinc acetate used to absorb the gas is slightly acidified with acetic acid, bringing the pH down to about 6·0 (Tarr, 1933). The other is that the mixture obtained by acidifying a solution of potassium tellurite with telluric acid absorbs H_2S at least as vigorously as the untreated solution.

There seems to be no necessity to assume that the mechanism which causes halo production differs in anything but degree from that which is responsible for the colouration of the colonies, either on the new medium or on TBA, for the proteins and peptone of TBA contain small amounts of cystine. If this is so, then the black colour of *mitis* and *intermedius* strains is due to the formation and retention of H_2S in the colonies while the different shades of grey in *gravis* colonies arise from the effect of the varying pH on the retention of H_2S within the colonies.

This ability to produce H_2S may also account, at least in part, for certain findings in clinical diphtheria. For example, the smell found in some cases may be due to the evolution of the gas from the cystine in the proteins of the membrane, and the blackening that results when the membrane is swabbed with potassium tellurite solution is probably due to the same cause.

SUMMARY

(1) A medium is described for the isolation and identification of *C. diphtheriæ*.

(2) The medium is at least equal to tellurite-blood-agar in the number of positive results obtained.

(3) The ease and certainty with which *C. diphtheriæ* can be identified is very much greater than with tellurite-blood-agar.

REFERENCES

ANDREWES, F. W., *et al.* . . . 1923. Medical Research Council, Diphtheria, *London.*
BULLOCH, W., *et al.* . . . . 1930. Medical Research Council, System of bacteriology, vol. v, *London.*
BÜRGER, M. . . . . 1914. *Arch. Hyg.,* lxxxii, 201.
DESNUELLE, P. . . . . . . 1939. *Enzymologia,* vi, 242.
PETRI, R. J., AND MAASSEN, A. . 1893. Arb. a. d. k. Gesundh. Amt., viii, 490.
TARR, H. L. A. . . . . . . 1933. *Biochem. J.,* xxvii, 759.
WILSON, W. J. . . . . . . 1934. This *Journal,* xxxviii, 114.

THE ROLE OF LEUKOTAXINE IN THE PRO-DUCTION OF THE ANHYDRÆMIA OF BURN SHOCK

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A polypeptide of comparatively low molecular weight and capable of causing increased capillary permeability has been described in inflammatory exudates from various sources (Menkin, 1940; Cullumbine and Rydon, 1946). This polypeptide was called "leuko-taxine" by Menkin and it appears to be liberated, after injury, at the site of the local damage. Its existence as such in the circulation is doubtful, since none has been detected in the blood of burned animals and it is known to be destroyed on incubation with blood serum or plasma (Cullumbine and Rydon). However, its local production at the site of injury might be a factor in the causation of the decreased blood volume and anhydræmia found in burn cases. The experiments reported here were designed to see if the systemic picture of anhydræmia could be reproduced by the localised injection of crude preparations of leukotaxine into the skin. In addition some preliminary observations on the toxic properties of the œdema fluids produced by the subcutaneous injection of leukotaxine and by burning have been made.

Methods

Rabbits were burned, under Nembutal anæsthesia, by exposing their depilated abdomens for 90 seconds to a glass vessel containing water at 70° C.

Two types of leukotaxine preparation were used, namely (i) that obtained after the peptic digestion of blood fibrin, and (ii) that obtained from the extraction of the burned skin of rabbits. In the latter case, the rabbits were killed four hours after burning and the burned areas of skin—each of about 150 sq cm —were immediately excised and extracted for leukotaxine. Simultaneous blood examinations indicated the presence of hæmoconcentration. Each burned area of skin yielded about 30,000 to 60,000 "units" of leukotaxine. The methods of extraction, partial purification and assay of activity have been described previously (Cullumbine and Rydon).

Red blood-cell counts, hæmoglobin estimations (Haldane), blood and plasma specific gravity determinations (copper sulphate method), total plasma-protein estimations, blood-urea estimations, and blood-glucose estimations (King, 1946) were made on the following groups of rabbits :—

(i) Rabbits each with about 150 sq. cm. of depilated abdominal wall burned as described.

(ii) Rabbits each with about 150 sq. cm. of depilated abdominal wall infiltrated, under Nembutal anæsthesia, with 15 c.c. of a solution of "fibrin leukotaxine" (60,000 "units") in distilled water.

(iii) Rabbits each with about 150 sq. cm. of depilated abdominal wall infiltrated, under Nembutal anæsthesia, with 15 c.c. of a solution of "skin leukotaxine" (60,000 "units") in distilled water.

(iv) Rabbits each with about 150 sq. cm. of depilated abdominal wall infiltrated, under Nembutal anæsthesia, with 15 c.c. of distilled water.

(v) Rabbits under Nembutal anæsthesia.

(vi) Rabbits receiving 15 c.c. of a solution of "fibrin leukotaxine" (60,000 "units") in distilled water intravenously.

Rabbits of 1·5 to 2·5 kg. body weight were used throughout and the dose of Nembutal used to produce anæsthesia was 30 mg./kg. intraperitoneally.

RESULTS

Anhydræmia in rabbits

The average results for each group of rabbits are given in tables I-VI and are shown graphically in figs. 1-4.

TABLE I

Effect of burning rabbits' abdomens at 70° C. for 90 seconds.   Average figures for 6 rabbits.   Burning done at Z hour

| Time (hours) | R.B.C. (per c.mm.) | Hb. (per cent.) | Specific gravity | | Plasma protein (g.per 100 c.c.) | Blood urea (mg. per 100 c.c.) | Blood sugar (mg. per 100 c.c.) |
			Blood	Plasma			
Z−24	4,740,000	58	1·047	1·021	5·4	26·8	98
Z	5,010,000	56	1·0465	1·021	5·4	27·4	105
Z+4	6,540,000	77	1·057	1·028	4·7	46·8	374
*Z+12	6,020,000	65	...	...	4·8	47·2	354
Z+24	5,070,000	55	1·047	1·018	5·0	45·1	120
Z+48	4,800,000	53	1·046	1·020	5·1	40·2	...

* Average of 3 rabbits.

TABLE II

Effect of subcutaneous injection of 60,000 units of "fibrin leukotaxine" into rabbits' abdomens.   Average figures for 6 rabbits.   Injection given at Z hour

| Time (hours) | R.B.C. (per c.mm.) | Hb. (per cent.) | Specific gravity | | Plasma protein (g. per 100 c.c.) | Blood urea (mg. per 100 c.c.) | Blood glucose (mg. per 100 c.c.) |
			Blood	Plasma			
Z−24	5,880,000	64	1·050	1·0214	5·5	28·1	107
Z	5,700,000	60	1·049	1·0216	5·6	30·3	117
Z+4	7,230,000	75	1·054	1·0210	4·8	38·0	251
*Z+12	6,650,000	70	...	...	4·8	45·1	235
Z+24	5,270,000	55	1·0465	1·021	5·1	46·3	133
Z+48	5,080,000	50	1·046	1·024	5·3	44·2	...
Z+96	4,790,000	48	1·045	1·0225	...	...	...

* Average of 3 rabbits.

It will be seen that the groups of burned rabbits and of those receiving "fibrin" or "skin leukotaxine" hypodermically show

TABLE III

Effect of subcutaneous injection of 60,000 units of "skin leukotaxine" into rabbits' abdomens. Average figures for 6 rabbits. Injection given at Z hour

Time (hours)	R.B.C. (per c.mm.)	Hb. (per cent.)	Specific gravity		Plasma protein (g. per 100 c.c.)	Blood urea (mg. per 100 c.c.)	Blood glucose (mg. per 100 c.c.)
			Blood	Plasma			
Z−24	6,040,000	60	1·047	1·020	5·2	27·6	105
Z	6,500,000	56	1·045	1·020	5·2	25·2	108
Z+4	7,290,000	72	1·0825	1·021	4·2	35·7	224
*Z+12	6,520,000	66	...	...	4·5	44·2	208
Z+24	5,020,000	52	1·043	1·020	4·6	44·6	125
Z+48	5,210,000	48	1·045	1·021	5·0	41·6	...
Z+96	4,890,000	46	1·043	1·0215	...	...	...

* Average of 3 rabbits.

TABLE IV

Effect of intravenous injection of 60,000 "units" of "fibrin leukotaxine" into rabbits. Average figures for 6 rabbits. Injection given at Z hour

Time (hours)	R.B.C. (per c.mm.)	Hb. (per cent.)	Specific gravity		Plasma protein (g. per 100 c.c.)	Blood urea (mg. per 100 c.c.)	Blood sugar (mg. per 100 c.c.)
			Blood	Plasma			
Z−24	5,960,000	61	1·040	1·0205	5·2	29·2	108
Z	5,830,000	61	1·048	1·021	5·5	24·3	112
Z+4	5,620,000	58	1·045	1·020	5·1	29·3	115
Z+24	5,520,000	59	1·049	1·021	5·5	31·5	110

TABLE V

Effect of subcutaneous injection of isotonic (0·9 per cent.) saline into rabbits' abdomens. Injection at Z hour. Mean of 6 rabbits

Time (hours)	R.B.C. (per c.mm.)	Hb. (per cent.)	Specific gravity		Plasma protein (g. per 100 c.c.)
			Blood	Plasma	
Z−24	5,500,000	55·0	...	...	...
Z	5,390,000	55·0	475	22·0	5·4
Z+4	5,430,000	50·5	480	21·5	5·2
Z+24	5,530,000	51·0	500	22·0	5·4
Z+48	5,700,000	51·0	510	21·0	5·1

substantially the same blood picture. The red blood-cell count, the blood-hæmoglobin content and the blood specific gravity all show a marked increase within four hours of injection or burning. This

increase is followed by a fall to lower than the pre-injection or pre-
burning levels.   Similarly both the burning of the skin and the
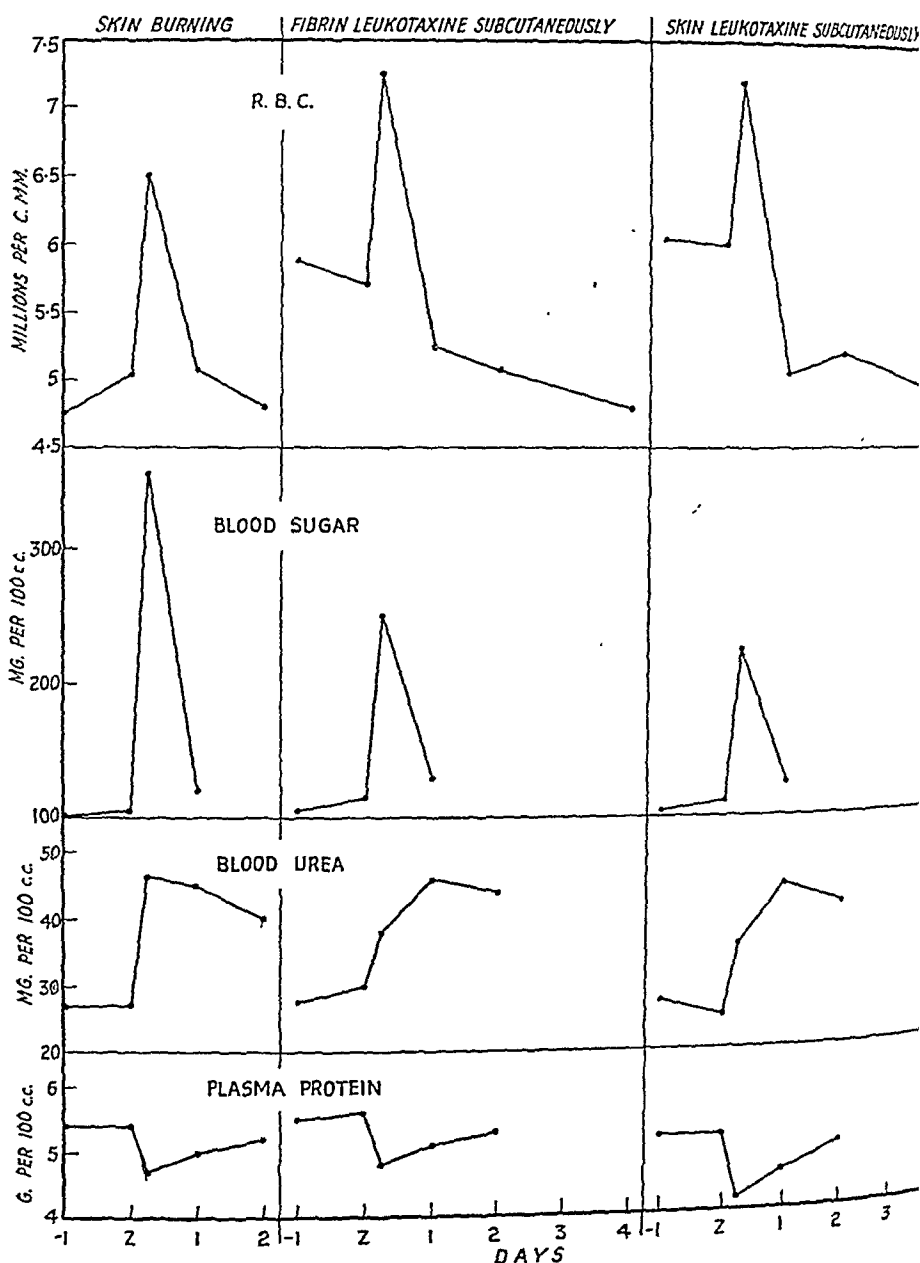
FIG. 1.—Effect on blood constituents of (a) skin burning, (b) fibrin leukotaxine (sub-
cutaneously), (c) skin leukotaxine (subcutaneously).

subcutaneous injection of leukotaxine are followed by a marked rise
in the blood-sugar level, a more sustained rise in the blood-urea level
and a fall in the plasma-protein content.

Control groups of rabbits receiving isotonic saline hypodermically or Nembutal intraperitoneally did not exhibit this blood picture.

TABLE VI

Effect of Nembutal anæsthesia (30 mg/kg. intraperitoneally at Z hour) on rabbits. Mean of 6 rabbits

Time (hours)	R B C (per c mm.)	Hb (per cent)	Specific gravity		Plasma protein (g per 100 c c)	Blood urea (mg per 100 c c)	Blood sugar (mg per 100 c c)
			Blood	Plasma			
Z−24	5,050,000	62	1 048	1 021	5 2	23 3	105
Z	4,980,000	60	1 048	1 0215	5 2	29 5	100
Z+4	4,520,000	57	1 046	1 020	5 0	26 9	112
Z+24	5,250,000	56	1 048	1 0205	5 1	29 3	108
Z+48	4,760,000	52	1 049	1 0205	5 1	31 7	.
Z+96	4,860,000	52	1 048	1 021	5 2	..	

Neither did a group which received 60,000 " units " of " fibrin leukotaxine " intravenously ; here, if anything, a picture of hæmodilution was produced.
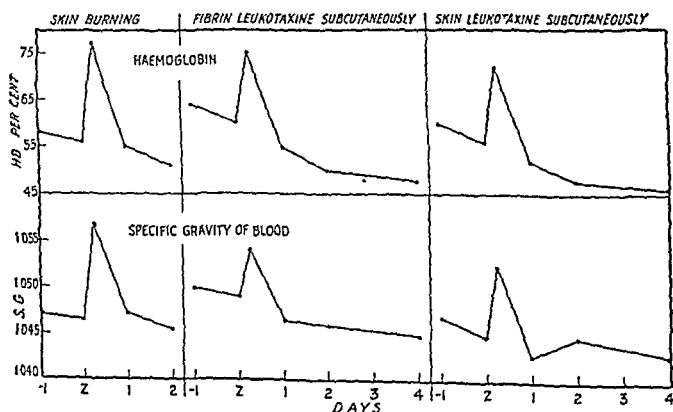
Fig 2 —Effect on the hæmoglobin content and *specific gravity* of the blood of (a) skin burning, (b) fibrin leukotaxine (subcutaneously) and (c) skin leukotaxine (subcutaneously)

The changes produced by the subcutaneous injection of the leukotaxine solutions were not due to their hypertonicity. Certainly the " fibrin leukotaxine " solutions were hypertonic, but the " skin leukotaxine " solutions, being purer preparations, were shown by direct measurement to be decidedly hypotonic.
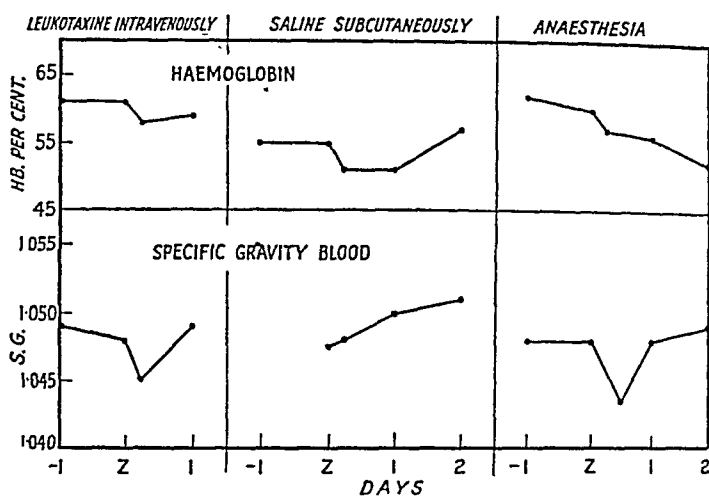
Fig. 3.—Effect on the hæmoglobin content and specific gravity of the blood of (a) intravenous leukotaxine, (b) subcutaneous saline and (c) anæsthesia alone.
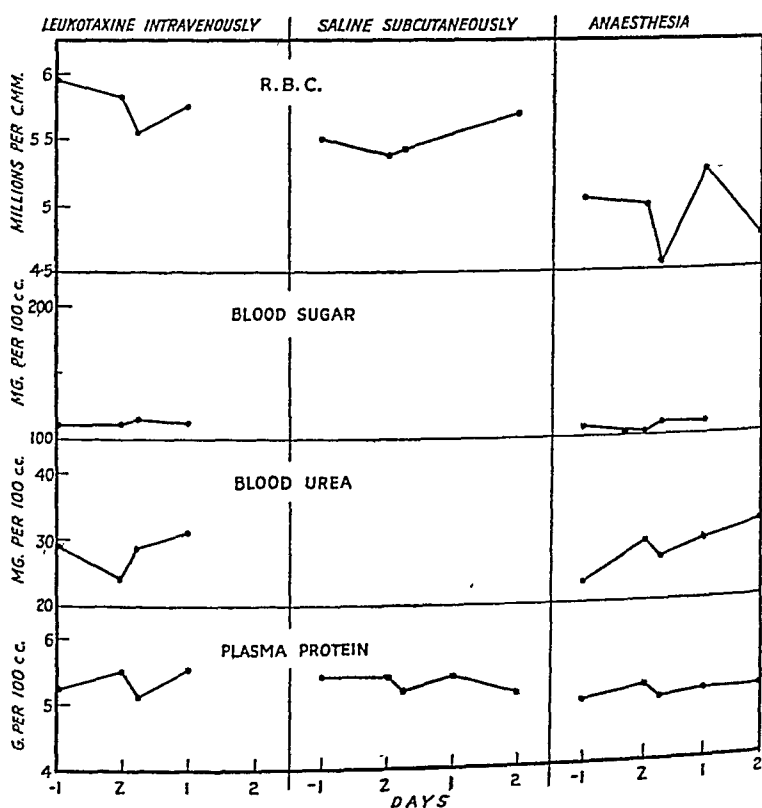
Fig. 4.—Effect on blood constituents of (a) intravenous leukotaxine, (b) subcutaneous saline and (c) anæsthesia alone.

Hence we can say that about 60,000 " units " of leukotaxine can be extracted from 150 sq. cm. of rabbit skin burned at 70° C. for 90 seconds, and that the subcutaneous injection of 60,000 "units" of this leukotaxine into normal rabbits causes marked local œdema and reproduces substantially the blood picture that follows burning of the skin.

Toxicity of burn œdema fluid

The production of leukotaxine in burn œdema fluid is not the whole story, however, in the pathogenesis of shock and death from cutaneous burning. Thus Wilson *et al.* (1936-37) have described the toxic properties of the œdema fluid obtained from the burned skin of rabbits. We have repeated their experiments and have confirmed their results. Eighteen rabbits under Nembutal anæsthesia were burned as before, six rabbits were killed at 4 hours, six at 24 hours and six at 48 hours after burning. The burned and œdematous areas of skin were excised, minced and filtered under pressure. The resulting fluids were injected intraperitoneally into mice and intravenously into normal rabbits. The results (table VII) show that, whereas

TABLE VII

Toxicity of œdema fluid obtained from rabbit skin after (a) burning at 70° C. for 90 seconds and (b) subcutaneous injection of 60,000 "units" of skin leukotaxine

Œdema fluid removed at	Toxicity to mice (intraperitoneal injection)		Toxicity to rabbits (intravenous injection)	
	Dosage in c.c./g.	48-hour mortality	Dosage in c.c./kg.	48-hour mortality
(a) **Burning at Z hour**				
Z+48 hours	0·01	0/10	10	3/4
"	0·025	7/10	...	...
"	0·05	10/10	...	...
Z+24 "	0·05	0/10	10	0/4
Z+4 "	0·05	0/10	10	0/4
(b) **Leukotaxine subcutaneously at Z hour**				
Z+48 hours	0·01	0/10	10	4/4
"	0·05	7/10	...	...
"	0·05	9/10	...	...
Z+24 "	0·05	0/10	10	0/4
Z+4 "	0·05	0/10	10	0/4

The leukotaxine solution (3200 "units" per c.c.), on injection intraperitoneally into mice, itself gave a 48-hour mortality of 1/10.

the fluids obtained at 4 and at 24 hours were without effect in the doses used, the 48-hour fluid was extremely toxic to both the mice and the rabbits.

Moreover, a fluid with similar toxic properties to mice and rabbits can be obtained from rabbit skin 48 hours after the subcutaneous injection of crude leukotaxine solutions (60,000 " units " per rabbit). The fluids obtained 4 and 24 hours after the subcutaneous injection of leukotaxine solutions were not toxic in the doses employed, nor were the original leukotaxine solutions.

The nature of the toxic factor in the 48-hour œdema fluid is at present under investigation. This fluid contains choline (0·4-0·7 mg. per c.c. by assay, after acetylation, on the isolated frog's rectus abdominis muscle) and histamine (3-4 μg. per c.c. by assay, after extraction by the method of Barsoum and Gaddum, 1935), but the 4- and 24-hour œdema fluids also contain comparable amounts of these bases.

DISCUSSION

A polypeptide, leukotaxine, can be extracted from the burned skin of rabbits. The subcutaneous injection of comparable quantities of leukotaxine into normal rabbits causes marked local œdema and reproduces substantially the blood picture which follows burning. This blood picture of hæmoconcentration, hyperglycæmia, etc., has been described after burning both in man and in the experimental animal (Davidson, 1925 ; Greenwald and Eliasberg, 1926 ; Beard and Blalock, 1931 ; McIver, 1933 ; Clark and Rossiter, 1943-44 ; Cameron *et al.*, 1945 ; etc.) and would seem to be typical of anhydræmia produced by a variety of means (Marriott, 1923 ; Govier and Greer, 1941 ; etc.). It is tempting to suggest that, after burning, one of the products of tissue damage may be a polypeptide capable of causing local increased capillary permeability, œdema, and so the systemic blood picture of anhydræmia.

The enzyme systems are, no doubt, affected and we have shown that the skin proteinase of Beloff and Peters (1944-45) can produce leukotaxine by the digestion of blood fibrin (Cullumbine and Rydon). Other workers have described the presence of proteolytic agents in the bleb fluid of human burns (Zamecnik *et al.*, 1945).

The suggestion that a toxin is liberated from burned tissue is, of course, not new ; Harkins (1938) listed twenty suggested toxins. Our leukotaxine preparations on intravenous injection in fairly substantial doses were apparently without effect. The only biological properties they appeared to possess were the production of increased capillary permeability and the migration of leucocytes at the site of their intracutaneous injection.

Leukotaxine cannot be the only factor in the production of shock and death from cutaneous burning. Wilson *et al.* described a toxic factor in burn œdema fluid and we have confirmed this. It would seem that the œdema fluid produced by the subcutaneous injection of leukotaxine contains a factor with a toxicity similar to that found after burning.

Summary

Leukotaxine can be extracted from the burned skin of rabbits. The subcutaneous injection of comparable quantities of leukotaxine into normal rabbits causes marked local œdema and reproduces substantially the blood picture which follows burning, while, just as after burning, the œdema fluid it provokes after 48 hours is highly toxic.

REFERENCES

BARSOUM, G. S., AND GADDUM, J. H.   1935. *J. Physiol.*, lxxxv, 1.

BEARD, J. W., AND BLALOCK, A.   1931. *Arch. Surg.*, xxii, 617.

BELOFF, A., AND PETERS, R. A. .   1044-45. *J. Physiol.*, ciii, 461.

CAMERON, G. R., ALLEN, J. W., COLES, R. F. G., AND RUTLAND, J. P.   1945. This *Journal*, lvii, 37.

CLARK, E. J., AND ROSSITER, R. J.   1943-44. *Quart. J. Exp. Physiol.*, xxxii, 279.

CULLUMBINE, H., AND RYDON, H. N.   1946. *Brit. J. Exp. Path.*, xxvii, 33.

DAVIDSON, E. C. . . . . .   1925. *Surg. Gyn. Obst.*, xli, 202.

GOVIER, W. M., AND GREER, C. M.   1941. *J. Pharmacol. and Exp. Therap.*, lxxii, 321.

GREENWALD, H. M., AND ELIASBERG, H.   1926. *Amer. J. Med. Sci.*, clxxi, 682.

HARKINS, H. N.. . . . . .   1938. *Surgery*, iii, 430.

KING, E. J. . . . .   1946. Micro-analysis in medical biochemistry, *London*, pp. 5, 21 and 37.

MARRIOTT, W. McKIM . . . .   1923. *Physiol. Rev.*, iii, 275.

MENKIN, V. . . . .   1940. Dynamics of inflammation, *New York*.

McIVER, M. A. . . . . . .   1933. *Ann. Surg.*, xcvii, 670.

WILSON, W. C., JEFFREY, J. S., ROXBURGH, A. N., AND STEWART, C. P.   1936-37. *Brit. J. Surg.*, xxiv, 601.

ZAMECNIK, P. C., STEPHENSON, MARY L., AND COPE, O.   1945. *J. Biol. Chem.*, clviii, 135.

SHORT ARTICLES

616 . 5—001 . 17 : 547 . 963 . 3

THE INFLUENCE OF CUTANEOUS BURNING AND LEUKOTAXINE ON THE ADENOSINE EQUIVALENT OF THE BLOOD OF RABBITS

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A polypeptide similar to Menkin's "leukotaxine" can be extracted from the burned skin of rabbits (Cullumbine and Rydon, 1946). The subcutaneous injection of leukotaxine into normal rabbits causes a marked local œdema and reproduces substantially the blood picture of hæmoconcentration, hyper-glycæmia etc., which follows burning and accompanies anhydræmia (Cullumbine *et al.*, 1947). Stoner and Green (1945) have shown that several different forms of bodily insult, including dehydration shock following the injection of hyper-tonic glucose, are accompanied in the rabbit by an increase in the adenosine equivalent of the blood. In the present paper we detail the results of experiments in which the adenosine equivalent of the blood of rabbits following cutaneous burning and the subcutaneous injection of leukotaxine was determined.

Methods

Rabbits of 1·5 to 2·5 kg. body weight were used. They were burned, under Nembutal anæsthesia, by exposing their depilated abdomens for 90 seconds to a glass vessel containing water at 70° C. Each rabbit had about 150 sq. cm. of the abdominal wall burned in this way.

In a further group of rabbits, about 150 sq. cm. of the abdominal wall of each animal was infiltrated, under Nembutal anæsthesia, with 15 c.c. of distilled water containing about 60,000 "units" of leukotaxine prepared by the peptic digestion of blood fibrin. Control groups of rabbits were similarly infiltrated, under Nembutal anæsthesia, with 15 c.c. of distilled water, or received Nembutal alone. The dosage of Nembutal used to produce anæsthesia was 30 mg./kg. intraperitoneally.

Blood samples were taken from the ear vein. Experimental and control animals were similarly treated. Hæmoglobin concentration (Haldane) and the adenosine equivalent of the blood (by the method of Drury *et al.*, 1937-38, after extraction according to Barsoum and Gaddum, 1935) were determined before and at various times after the burning or injection.

Results

The group of rabbits suffering from cutaneous burns showed an average increase of 27 per cent. in the blood level of the adenosine equivalent (minimum increase 20 per cent., maximum 38 per cent., table I). A group of rabbits bled at similar times and anæsthetised with Nembutal in a similar manner showed an average increase of only 7 per cent. in the level of the adenosine equivalent (table II). The increase shown by the group of burned rabbits is significant at P = 0·05.

Rabbits injected subcutaneously with an aqueous solution of leukotaxine showed an average rise of blood-adenosine equivalent of 24 per cent. (minimum

TABLE I

Effect of burning on the level of the adenosine equivalent in the blood

Rabbit no.	Adenosine equivalent (μg. per c.c. whole blood) corrected for Hb. per cent.						
	Before burning		After burning (time in hours)				
			0·5	1	2	4	6
1	250	275	310	320	300	280	270
2	230	210	310	250	250	210	200
3	280	300	320	360	310	290	300
4	290	280	400	350	320	330	300
5	310	340	390	410	380	320	290
6	190	230	250	300	300	220	210

TABLE II

Effect of Nembutal (30 mg./kg.) anæsthesia and of subcutaneous distilled water on the adenosine equivalent of the blood

Rabbit no.	Treatment	Adenosine equivalent (μg. per c.c whole blood) corrected for Hb. per cent.						
		Before injection		After injection				
				0·5	1	2	4	6
13	Nembutal	190	210	220	230	200	210	200
14	,,	280	270	300	310	290	260	280
15	,,	320	290	310	300	280	290	280
16	Water	240	240	260	270	240	220	250
17	,,	220	250	220	260	240	240	230
18	,,	270	290	310	320	300	280	270

TABLE III

Effect of subcutaneous leukotaxine on the adenosine equivalent of the blood

Rabbit no.	Adenosine equivalent (μg. per c.c. whole blood) corrected for Hb per cent.						
	Before injection		After injection (time in hours)				
			0·5	1	2	4	6
7	300	320	410	370	360	300	300
8	330	280	390	360	360	320	290
9	250	260	270	320	310	300	240
10	270	270	310	390	380	280	200
11	310	300	350	380	360	300	320
12	290	290	320	310	280	290	300

increase 7 per cent., maximum 44 per cent. ; table III). Each of these rabbits received 60,000 units of leukotaxine subcutaneously and we had found previously (Cullumbine *et al.*) that 30,000-60,000 units of leukotaxine can be extracted

from 150 sq. cm. of the abdominal skin of rabbits after cutaneous burning at 70° C. for 90 seconds.

A control group of rabbits receiving a subcutaneous injection of distilled water while under Nembutal anæsthesia showed a mean rise of blood-adenosine equivalent of 9 per cent. The rise occurring in the leukotaxine-injected rabbits is significant at P = 0·05.

DISCUSSION

These experiments show, therefore, that the cutaneous burning of rabbits provokes an early increase in the adenosine equivalent of the blood. About 30,000-60,000 units of leukotaxine can be extracted from the burned area (skin and œdema fluid) of such rabbits, and the subcutaneous injection of 60,000 units of leukotaxine into normal rabbits causes an increase in the adenosine equivalent of the blood similar to that produced by burning.

The increases of blood-adenosine levels here reported are less than those observed by Stoner and Green, but in their experiments the rabbits were subjected to a rapidly fatal degree of trauma (survival less than 6 hours), whereas our rabbits survived for 2-4 days after the experiment.

If these results are added to the findings of Cullumbine *et al.* it is evident that in rabbits substantially the same picture can be produced in two different ways, the first by burning the skin of the animals and the second by injecting them subcutaneously with amounts of leukotaxine comparable to those extracted from the burned skin. Both give rise to œdema fluid containing similar amounts of choline (burning 0·4-0·7, leukotaxine 0·3-0·65 mg. per c.c.) and histamine (burning 3-4, leukotaxine 2·5 mg. per c.c.) ; in both, Wilson's toxic factor (Wilson *et al.*, 1936-37) is present in the 48-hour and absent from the 4- and 24-hour œdema fluids ; and both give the same blood picture, with increases in the hæmoglobin, red-cell count, specific gravity, blood urea, blood sugar and adenosine equivalent.

SUMMARY

The adenosine equivalent in the blood of rabbits is raised to similar levels by burning the skin of the animals and by injecting them subcutaneously with amounts of leukotaxine comparable to those extracted from the burned skin.

Other points of similarity noted in previous experiments are summarised in the discussion.

REFERENCES

BARSOUM, G. S., AND GADDUM, J. H. 1935. *J. Physiol.*, lxxxv, 1.

CULLUMBINE, H., McDONALD, F. F., AND SIMPSON, M. M. 1947. This *Journal*, lix, 467.

CULLUMBINE, H., AND RYDON, H. N. 1946. *Brit. J. Exp. Path.*, xxvii, 33.

DRURY, A. N., LUTWAK-MANN, C., AND SOLANDT, O. M. 1937-38. *Quart. J. Exp. Physiol.*, xxvii, 215.

STONER, H. B., AND GREEN, H. N. 1945. This *Journal*, lvii, 337.

WILSON, W. C., JEFFREY, J. S., ROXBURGH, A. N., AND STEWART, C. P. 1936-37. *Brit. J. Surg.*, xxiv, 601.

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AN IMPROVED TECHNIQUE FOR INOCULATION OF FERTILE EGGS *

L. HOYLE

From the Public Health Laboratory, Northampton

The technique described in this paper was developed to overcome certain difficulties experienced in performing inoculations of the amniotic sac by the method of Burnet (1940). Inoculations can be made with great precision and under direct observation to any desired portion of the egg contents.

Inoculation of the amniotic sac

Twelve-day-old fertile eggs are used. The narrow end of the egg is sterilised with a suitable antiseptic (*e.g.* 0·5 per cent. brilliant green+0·5 per cent. crystal violet in 50 per cent. alcohol) and a circular cut is made half-way through the shell round the narrow end, the circle being 1·5-2·0 cm. in diameter. A small square window (0·5 cm.) is then cut in the centre of the circle at the extreme tip of the egg and the square of egg shell removed without damaging the egg-shell membrane. A small hole is then made at the broad end of the egg into the air sac. The egg is now placed with the narrow end upwards and the egg-shell membrane in the centre of the square window perforated with a needle without damaging the underlying chorio-allantoic membrane. Suction is now applied to the hole at the broad end and air enters at the narrow end; the chorio-allantoic membrane separates from the shell and an artificial air space is created. The egg shell is then broken away to the limits of the circle first marked out, thus creating a circular window 1·5-2·0 cm. in diameter. The egg is now illuminated by a powerful beam of parallel light 1 inch in diameter directed at the side of the egg. A Beck intensity lamp such as is used for microscope illumination is suitable. The whole contents of the egg can then be clearly seen through the window, since the exposed area of chorio-allantoic membrane is very thin, transparent and free from large vessels. Inoculations can now readily be made into the chorio-allantoic membrane, the allantoic sac, the yolk sac, the amniotic sac, or the embryo itself. For inoculation of the amniotic sac a Pasteur pipette is drawn out very fine and broken off to give a sharp end. The inoculum (0·1 c.c.) is drawn into the pipette, which is then passed through the chorio-allantoic membrane and driven with a sharp stab through the amnion. The inoculum is introduced and followed by a small bubble of air which, if the inoculation is correct, can be clearly seen lying in the amniotic sac. The window is now closed with a small metal cap 2·0-2·5 cm. in diameter dipped in molten paraffin wax. The metal caps of ordinary screw-capped bottles are very suitable. The egg is then incubated with the cap upwards.

The success of the technique depends on the use of a sufficiently powerful lamp to illuminate the egg and on the use of an extremely fine and sharp pipette to make the inoculation. In a small proportion of eggs the narrow end is entirely occupied by the yolk sac and direct inoculation of the amniotic sac is not possible, but if a small slit is made in the chorio-allantoic membrane a pair of fine curved forceps can be introduced, the amnion seized and drawn up in a manner analogous to Burnet's technique, and the inoculation made.

By removing the cap the egg contents may be inspected daily during the course of incubation with the aid of the lamp, and samples of allantoic or amniotic fluid may be removed. The technique has been very successful in work with influenza viruses.

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* A report to the Medical Research Council.

REFERENCE

Burnet, F. M   .   .   .   1940. *Austral. J. Exp. Biol. Med. Sci.*,
xviii, 353.

616—006 . 384—021 . 6 (Cuniculus) : 547 . 689 (cholanthrene)

AN UNSUCCESSFUL ATTEMPT TO INDUCE GLIOMATA IN
RABBITS WITH CHOLANTHRENE

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(Plate LXX)

This experiment was the sequel to a study of human gliomata (Russell, 1941) and its object was to find out what types of tumour growth might develop in the brain of the rabbit in response to the presence of a carcinogen.

The polymorphism of human gliomata is well known and has led to the remarkable histological subdivisions inaugurated by Bailey and Cushing (1926).

Experimental attempts to induce gliomata in the mouse brain by the implantation of carcinogens have resulted in tumours which have been considered analogous to the human types and with as wide a range of variation in morphology Various carcinogens were employed , methyl cholanthrene by Seligman and Shear (1939) and Zimmerman and Arnold (1941), and 1 . 2 . 5 . 6. dibenzanthracene contained in cholesterol pellets by Peers (1940) Experiments with rats and rabbits have been less numerous and less successful (Bertrand and Gruner, 1938, using 1 : 2 benzpyrene , and Oberling *et al*, 1936, using 3 4 benzpyrene)

It was hoped that the rabbit brain, being larger than that of the mouse, might provide a more suitable medium in which to study neoplasia , further, it is very easy to implant material into the cerebral cortex of the rabbit without fear of inducing more than a minimal mesodermal reaction from the meninges and tissues of the scalp On the other hand it was realised that rabbits do not seem to respond to carcinogens so readily as mice, and that, as their span of life is greater, a longer experiment would be necessary.

The experimental details are summarised in the accompanying table. The rabbits were divided into experimental and control groups , the former being implanted with cholanthrene—a known carcinogen, the latter with anthracene, which has no carcinogenic activity. These substances were used in pure form compressed into pellets of about 20 mg. weight By this means chemical injury to the brain from organic solvents and the complication of phagocytic reaction around fat solvents were avoided. Three sites were chosen for the implants— the cerebral cortex, the eye and the cerebellum. The cerebral cortex implant is by far the easiest operation and the results in that group are those to which most attention was paid In the cerebellar operation bleeding may be so profuse that the pellet becomes displaced, and in the eye operation the 20 mg pellet was too large for a safe trephine hole and smaller doses had to be used.

The animals were bought in the open market in 1941 and varied in age, weight and breed. Most of them were infected with coccidiosis and a few had helminthic infections , their blood counts were as variable as was to be expected in such a mixed group At intervals during the four and a half years of

the experiment some of the animals died of intercurrent disease but about half the original number were fat and well at the end of 1945 when the experiment was ended. The results were negative; no indication of tumour growth was found in any of the rabbits at any time.

TABLE

Details of an unsuccessful attempt to induce gliomata in rabbits

Rabbit no.	Site of implant	Pellet of cholanthrene or anthracene (wt. in mg.)	Survival after implant	
EXPERIMENTAL GROUP (CHOLANTHRENE)				
218	Cerebrum	20	4 yrs.	Died
220	,,	20	4½ ,,	Killed
222	,,	20	4 months	Died
224	,,	15	4½ yrs.	Killed
226	,,	15	4¼ ,,	Died
228	,,	15	4½ ,,	Killed
230	,,	. 15	4¼ ,,	Died
232 *	,,	20	4½ ,,	Killed
234	,,	20	15½ months	Died
236	,,	20	4½ yrs.	Killed
238	,,	20	9½ months	Died
240	Eye	c. 4	4½ yrs.	Killed
242	,,	c. 4	5 months	Died
244	,,	c. 4	4½ yrs.	Killed
246	,,	c. 4	3½ ,,	Died
262	Cerebellum	20	4½ ,,	Killed
264	,,	20	4 1/12 ,,	Died
266	,,	c. 12	4½ ,,	Killed
268	,,	c. 15	4¼ ,,	Killed
270	,,	c. 12	3 7/12 ,,	Died
CONTROL GROUP (ANTHRACENE)				
213	Cerebrum	20	20 months	Died
215	,,	20	4 yrs.	Died
217	,,	20	4½ ,,	Killed
219	,,	10	4¼ ,,	Died
221	,,	20	2 5/12 ,,	Killed
241	Eye	c. 4	4¼ ,,	Killed
243	,,	c. 5	4½ ,,	Killed
263	Cerebellum	c. 12	4¼ ,,	Died
265	,,	c. 12	4¼ ,,	Killed

* See figs. 1 and 2.

There is little to be said about the histological reaction of the nervous tissue to the presence of either anthracene or cholanthrene in this form. The pellets were implanted aseptically and remained in the brain as immobile foreign bodies, generating around them minimal glial reaction (figs. 1 and 2) in which mesodermal giant cells were sometimes recognised.

These negative observations are published in order to emphasise the importance of factors other than the carcinogen in neoplasia, factors which are not only species specific but which may even have a limited distribution within a given species.

EXPERIMENTAL CARCINOGENESIS
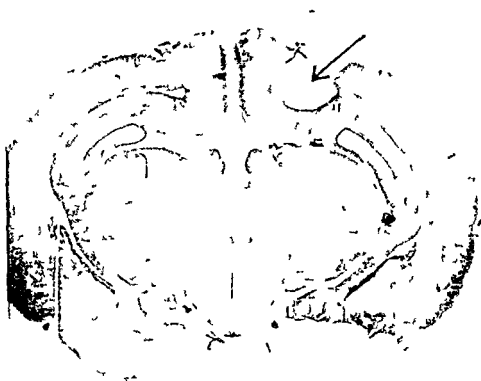
Fig 1—Section of brain in rabbit 232 showing the pocket of reaction formed in the cerebral cortex around a cholanthrene pellet which had been implanted 4½ years before   × 5
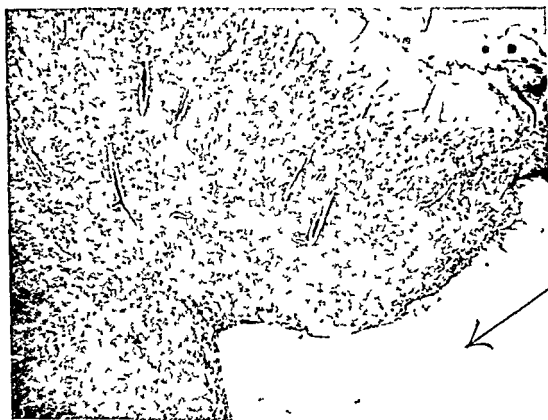
Fig 2—Higher power view of part of the wall of the pocket which contained the cholanthrene pellet, showing minimal glial reaction   × 50

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Summary

This paper is the record of an unsuccessful attempt to induce gliomata in rabbits by implanting a pellet of cholanthrene into parts of the nervous system. No tumours developed, although about half of the animals survived for four and a half years after the implantation had been carried out.

I have to thank Professor Murray Drennan for allowing me to undertake this long experiment in his department, Dr William Blackwood for examining specimens from animals which died while I was away from Edinburgh, and Dr A. Haddow for the cholanthrene and anthracene pellets.

REFERENCES

BAILEY, P., AND CUSHING, H.	1926.	A classification of the tumors of the glioma group, *Philadelphia*.
BERTRAND, I., AND GRUNER, J.	1938.	*Bull. de l'Assoc. p. l'étude du Cancer*, xxxvii, 697.
OBERLING, C., GUÉRIN, M., AND GUÉRIN, P.	1936.	*Compt. rend. Soc. de biol.*, cxxiii, 1152.
PEERS, J. H.	1940.	*Amer. J. Path.*, xvi, 799.
RUSSELL, H.	1941.	*Edin. Med. J.*, xlviii, 145.
SELIGMAN, A. M., AND SHEAR, M. J.	1939.	*Amer. J. Cancer*, xxxvii, 364.
ZIMMERMAN, H. M., AND ARNOLD, HILDEGARDE	1941.	*Cancer Research*, i, 919.

EXTENSIVE CARCINOMATOUS CHANGE IN A CASE OF CHRONIC ULCERATIVE COLITIS

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The incidence of malignant change in cases of chronic ulcerative colitis has been the subject of many communications in the literature of the United States, but no paper has been found in a search of English journals covering the past twenty-five years. This case is reported briefly, for it presents most of the features said to be characteristic of the condition, and so may lead to a better recognition of this interesting association.

Case report

Clinical history. Mrs G. P., aged 27 years, was admitted to the General Infirmary at Leeds on 20.8.46 complaining of recurrent lower abdominal pain and diarrhœa. She stated that she had first suffered from an attack of diarrhœa in 1938 when, at the age of 18 years, she was confined to bed under medical care for six weeks. Since that time she has been subject to recurring attacks lasting about a month, followed by remissions of variable length, the last and longest of which was of about one year's duration. The attacks consisted in passing about ten *watery stools in 24 hours*; only on rare occasions had she noticed any mucus or blood. Her appetite remained good in these relapses, and she was not much inconvenienced, for over the years she had been married and had borne a son who is now four years of age and healthy.

In 1944 she attended the out-patient department, when a barium enema showed the typical changes of ulcerative colitis.

During the month which preceded her admission on the present occasion she had experienced the worst attack to date, and for the first time there was severe pain, always in the lower abdomen. Examination showed that she was rather wasted—her best weight had been 10 st., and she was now 7 st. 3 lb. Her finger nails were bitten to the quick. General examination revealed nothing of note. Rectal examination showed the presence of a polypoidal mass in the ampulla. It surrounded the lumen of the gut and was neither tender nor fixed. A biopsy showed an adenomatous polyp with slight irregularity of the cells, but apparently benign. There was considerable chronic inflammatory-cell infiltration. A further barium examination on 31.8.46 merely confirmed the previous report.

Temporary improvement followed and the patient was able to go home. On 25.10.46 she was readmitted with much lower abdominal pain and incontinence of fæces. After preliminary transfusion she was submitted to laparotomy, when extensive (? neoplastic) involvement of the large intestine was noted, and a de Pezzer catheter was inserted into the cæcum. Death occurred 24 hours later.

Post-mortem findings. A post-mortem examination was made on 7.11.46 by Professor M. J. Stewart. The main findings were as follows.

On opening the peritoneal cavity there was found a localised abscess between the sigmoid colon and the left postero-lateral abdominal wall. It was effectively sealed off from the peritoneal cavity by the great omentum.

The cæcum, ascending colon and transverse colon were the seat of advanced ulcerative colitis. In the cæcum and ascending colon the lesion was hæmorrhagic, and here very little mucosa remained. In the region of the splenic flexure there was a sharp change in the character of the lesion. For about six inches there were numerous polypoidal projections from the mucosa. Below this there was frank malignancy, confined to the mucosa in the sigmoid colon, but in the rectum there was a cancerous mass some 3 cm. in thickness encircling the viscus and involving the whole thickness of the wall. The abscess noted earlier arose from a perforation of the sigmoid colon. The malignant change ceased about 8 cm. from the anus, and this last portion of the bowel showed two apparently independent polypoidal masses. Small neoplastic deposits were present in the peritoneum of Douglas's pouch and there was evident invasion of regional lymph glands. The small intestine was normal throughout.

Incidental findings were a thyroid gland which showed moderate lymphadenoid change, a liver which was the seat of irregular patchy scarring like that seen after hepatic necrosis, although there was no history of hepatitis; this lesion was proved histologically to be old and well healed.

Histology. Histological examination of portions of the affected bowel showed that the malignant area was a glandular, mucus-forming carcinoma and the deposits in the lymph glands were of the same type. The portion of the bowel which had not undergone malignant change showed typical superficial ulcerative colitis with much fibrosis of the submucosa. There was no involvement of the muscle in the parts examined.

Discussion

In England at least the change from simple ulceration to malignancy in cases of chronic ulcerative colitis does not appear to be a common one. No case record has been found in the literature of this country in the past 25 years, and the condition is not mentioned in Stewart's Croonian Lectures (1931). Hurst (1946), in his article in Price's *Textbook of medicine*, states that the small polypi which may form during the healing of chronic ulcerative colitis may

become malignant. By contrast, there is an extensive American literature on the subject, the main writer being J. A. Bargen of the Mayo Clinic (Brust and Bargen, 1934 ; Bargen *et al.*, 1938-39). There are in fact many published series, one of them containing as many as nine cases (Cattell and Boehme, 1946). Bargen considered the reports of 871 proved cases of chronic ulcerative colitis —491 males and 380 females—and found that the incidence of malignant change was 3·2 per cent., a figure which is the more striking when one realises that the incidence of cancer of the colon revealed in the vital statistics of the United States for the same period was 0·011 per cent.

A consideration of the main articles and the detailed case reports makes it clear that there are some characteristic features of this condition, many of which are exhibited by the case here recorded. (1) The average duration of symptoms before the onset of malignancy is about nine years. (2) Malignant change is seen most often in those whose symptoms begin in childhood or in young adult life. (3) Different parts of the bowel are usually affected simultaneously and the malignancy is of high grade. (4) The malignant change is heralded by a change in symptoms which, previously, had often been constant in character for years ; the course thereafter proceeds rapidly downhill.

There would appear to be a difference in the incidence of this association in the United States and in this country, for there can be little doubt that the condition is rare in Great Britain. The post-mortem records of the General Infirmary at Leeds have been examined since 1910, and although there are 72 cases of ulcerative colitis recorded, no case of malignant change was noted in any of them. The incidence of carcinoma of the intestine, excluding duodenum and rectum, in England and Wales for the ten years 1932-1941 was 0·015 per cent., which agrees closely with the findings in the United States. It may well be that the majority of these cases die in their own homes after a long illness and so do not come to autopsy. Once malignant change has taken place the outlook would appear to be hopeless in the great majority of cases, but it may be possible to determine that a certain type of case is especially prone to pursue this course. If so, there may be justification for some of the heroic surgical measures hitherto employed, which, in recent years, have rather fallen into disrepute.

Summary

1. A case of chronic ulcerative colitis is reported in which carcinomatous change affecting a long reach of the large bowel occurred.

2. The lack of reports of such cases in the English literature and their relative frequency in that of the United States is noted.

3. Attention is drawn to the more characteristic features of the reported cases : the long duration of the colitis before the malignancy supervenes, the early age incidence and the high-grade malignancy once it has appeared.

I should like to express my thanks to Professor Matthew Stewart for the suggestion to publish this case report and for his assistance in the preparation of the paper.

REFERENCES

BARGEN, J. A., JACKMAN, R. J., AND KERR, J. G.   1938-39. *Ann. Int. Med.*, xii, 339.

BRUST, J. C. M., AND BARGEN, J. A.   1934. *New England J. of Med.*, ccx, 692.

CATTELL, R. B., AND BOEHME, E. J.   1946. *Surg. Clin. of N. Amer.*, xxvi, 641.

HURST, A. F.   .   .   .   .   .   .   1946. *In* Price's Textbook of the practice of medicine, 7th ed., *London*, p. 682.

STEWART, M. J.   .   .   .   .   .   1931. *Lancet*, ii, 617.

578.6

A NEW WAY OF APPLYING IMMERSION OIL

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For over seventy years now microscopists have dipped rods into bottles of immersion oil and tried to transfer a suitable drop to a microscope slide or a lens surface. It is an awkward method and may have persisted merely because microscope makers supply oil bottles and rods free.

The inherent shortcomings of the method became manifest when I was working with a microscope in a nearly horizontal position. This necessitated the introduction of large drops of oil both between the darkground condenser and slide and between the coverslip and objective, because gravity combined with movement of the slide caused loss of contact unless the original drop was large. Getting such large drops into place called for skilled and quick manipulation.

Accordingly I looked round for a better method, and now employ a dropping bottle as illustrated (fig.). Instrument catalogues list it as a Schuster bottle and its volume is about 30 c.c., but it works best with only a few c.c. of oil. The oil is poured in through the opening on the right, which is then closed with a cork. A glass stopper would become sticky and require cleaning.

3½″

FIG.—Schuster bottle.

To deliver a drop of oil, the bottle is tilted so that the oil runs slowly into the thin spout on the left, giving time to bring the tip of the spout to the spot where the drop is to be deposited. Several drops can be got out in rapid succession with a single tilting. The bottle is returned to the upright position as soon as enough oil has been deposited. The longer the tip is kept down, the bigger the drop becomes. Righting the bottle makes the oil flow back at once to the bottom of the bottle and none is left in the spout. With ordinary care the oil does not flow over to the outside of the tip and none is wasted.
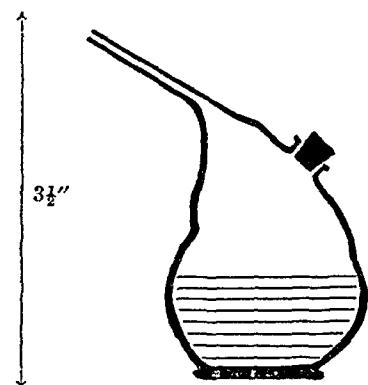
Further advantages may be named : air bubbles are automatically excluded ; cleanliness is favoured ; the long and narrow spout prevents evaporation, so that the oil does not thicken ; the spout never gets blocked ; and the bottle is ready for use at any time, even after standing unused for several days. For longer periods of idleness one may push a tiny cork over the tip. The dangers of scratching lenses or rubbing off bits of the microscopic preparation are eliminated, because the oil flows freely and so there is no need to get too near these surfaces.

In short, the rod-and-bottle method can with advantage be replaced by this dropping bottle. It is a handmade article, and individual bottles show slight variations in the width of the spout. An inside diameter of about 1/16 inch (1·5 mm.) suits me for Gurr's " Microil " ; for colder climates or thicker oil one might choose a bottle with a slightly wider spout.

576 . 851 . 21 (*Str. hæmolyticus*) : 576 . 8 . 095 . 5

THE PRODUCTION OF *VIRIDANS* VARIANTS OF HÆMOLYTIC STREPTOCOCCI

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There are many references in the literature to the relationship between hæmolytic and *viridans* streptococci (the α type of Brown, 1919). While they are often regarded as distinct species, a number of workers have suggested that the two forms are interchangeable. The clinical significance of this is indicated by the findings of Coburn and Pauli (1941), who reported an epidemic of streptococcal infections in children caused by virulent type-12 hæmolytic streptococci, contagion being spread by carriers of type-12 " non-hæmolytic " streptococci. The production of *viridans* forms from hæmolytic streptococci has been claimed by some workers and denied by others (see review by Brown). Animal passage, growth of the organisms in immune sera, and the action of Optoquine or Rivanol mixed with yeast have been claimed to produce these variants. Thus Todd (1928) studied a hæmolytic streptococcus from a case of puerperal sepsis which could always be rendered non-hæmolytic by passage through four or five mice. The passage culture differed from typical strains of *Streptococcus viridans*, however, in showing hæmolysis in anaerobic growth and reversion to the hæmolytic form on prolonged subcultivation in artificial media.

During work on the viability of streptococci cultured under raised oxygen tension, it was observed that a *Streptococcus pyogenes* of human origin gave rise to a proportion of variant colonies which showed a *viridans* appearance on blood agar and retained that character on repeated subcultivation over a period of six months ; 15 strains were investigated in all, and in 9 of these the phenomenon was observed. The method appears to be a novel one and as it has yielded α variants in a high proportion of the strains studied, details of the method and the characters of the variants are described.

Methods

Strains were isolated from pus from septic lesions and some were obtained from the National Collection of Type Cultures : 9 strains belonged to Lancefield's group A, 2 were group C, 2 group E and one each belonged to groups G and L. All were actively hæmolytic on blood agar and produced hæmolysin in serum-containing media. Prolonged examination involving many hundred platings in the case of several strains showed only hæmolytic colonies. Each strain was first plated twice and a strongly hæmolytic colony picked.

The medium was horse-heart infusion broth, and originally 10 per cent. of oxalated normal horse blood was added, but this was not essential ; 5 c.c. amounts were placed in 100 c.c. Erlenmeyer flasks. One of these was inoculated with a colony of the strain to be tested and put in an anaerobic jar through which oxygen from a cylinder was passed for 20-30 minutes and then incubated at 37° C. The flask contents were plated daily on 7½ per cent. horse-blood agar, and the flask again exposed to an oxygen atmosphere. The first day's plating yielded only hæmolytic colonies. After a further 2 or 3 days, the colonies growing in subcultures were less strongly hæmolytic and the broth culture gradually died out. One of the least hæmolytic colonies was used to inoculate a second flask of broth and the procedure repeated until *viridans* variants

* McCunn Research Scholar.

appeared on plating. Most strains gave such variants within 4 or 5 passages, but with one strain 25 passages were required. Especially if smooth colonies were selected for passage, variants were readily obtained which continued to produce only *viridans* organisms on further cultivation in broth or glucose-broth exposed to air.

Characters of the variant strains

Cultural and microscopic characters. *Viridans* colonies, while generally of the same size as the parent hæmolytic colonies, were always smooth or glossy, and grew in fluid medium as a uniform suspension of short chains. The transitory appearance in 5 strains of a diplococcal phase was striking. The colonies on blood agar were glossy, larger than those of the original hæmolytic strain and with a very wide zone of greening, fully 6 mm. in diameter; in fluid medium growth formed a uniform suspension. The cocci were large, strongly Gram-positive and non-capsulated. On continued subcultivation in air or under raised oxygen tension, the chaining form was regained in 3 strains and the usual *viridans* colonies appeared. In two, however, the diplococcal form persisted indefinitely.

The variants required frequent subcultivation for maintenance as compared with the original strain. Growth failed in media such as 1 per cent. peptone water, which supported growth of the parent hæmolytic strains.

Biochemical reactions. The *viridans* variants often showed weak fermentation reactions as compared with the parent strains. Thus 3 lactose-fermenting hæmolytic strains yielded *viridans* variants which did not ferment lactose. None of the original hæmolytic strains and only one variant gave a positive Voges-Proskauer reaction (Lominski, Harper and Isaacs, 1946), but most of the variants grew poorly in glucose-phosphate peptone water. All the *viridans* variants produced large amounts of peroxide in broth, even where the corresponding hæmolytic strains failed to give a positive benzidine test. In addition, the variants, and particularly the diplococcal forms, caused marked bleaching on chocolate agar. Like the parent strains, none produced catalase.

Hæmolysin production. On blood-agar plates incubated aerobically the variants showed greening, and under anaerobic conditions greening with no trace of hæmolysis. Neither streptolysin O nor S (Todd, 1938) were produced by the variants.

Serology. Rabbits were immunised by the method of Lancefield (1933) with formol-killed broth cultures of three hæmolytic strains of groups A, C and E, and the corresponding *viridans* variants. On testing by the micro-precipitin method of Lancefield (1938), the resulting antiserum in each case gave a precipitate with an antigen prepared from the homologous strain but not from the corresponding variant. An example of these results for a group-A strain and its variant is given in the table.

TABLE

Cross-precipitation test between original hæmolytic (β) form and induced viridans (α) *variant of Strep. pyogenes*

Strain	Antigen	Antiserum to	
		β	α
Group A	β form	+	−
	α variant	−	+

None of the variants, as tested with commercial antisera, possessed the group-specific antigen of either the parent hæmolytic strain or of heterologous groups. Accordingly, in the course of variation the carbohydrate antigen (" C " substance of Lancefield) has been lost or modified in some way.

Reversion to hæmolytic form. This occurred in a proportion of the colonies of only two of these *viridans* variants. With one, many subcultures were carried out over two months before the reversion took place, the group-C carbohydrate antigen of the original hæmolytic strain being also regained. In the other, reversion occurred shortly after the variant was isolated.

Discussion

The stability of the hæmolytic property of streptococci under ordinary conditions of culture has been emphasised by many workers, who found that it persisted over years in artificial media (Brown). The question arises whether the development of the *viridans* forms under raised oxygen tension merely favours development of variants by a process of selection, as it were, or whether there is a gradual loss of hæmolysin production with replacement by the *viridans* characters. While the methods of the present work do not afford a definite answer, there was clear evidence in several strains of a number of stages from hæmolytic and rough to *viridans* and smooth.

It is of interest that in the variant strains, the loss of capacity to form hæmolysin and group-specific carbohydrate antigen is associated with gain in the active production of peroxides. It appears that oxidation is directly responsible for the changed characters of the organisms. Experimental evidence of this will be reported shortly.

Summary

1. A method has been described for inducing *viridans* variants of hæmolytic streptococci *in vitro*. It involves growth under raised oxygen tension and was successful in nine out of fifteen strains tested.

2. The cultural and antigenic characters of the variants are described and their relationship to the parent hæmolytic forms discussed.

REFERENCES

BROWN, J. H.	1919.	Monographs of the Rockefeller Institute for Medical Research, no. 9, *Washington.*
COBURN, A. F., AND PAULI, R. H.	1941.	*J. Exp. Med.,* lxxiii, 551.
LANCEFIELD, REBECCA C. . .	1933.	*Ibid.,* lvii, 571.
,, ,, . .	1938.	*Proc. Soc. Exp. Biol. and Med.,* xxxviii, 473.
LOMINSKI, I., HARPER, E. M., AND ISAACS, A.	1946.	This *Journal,* lviii, 607.
TODD, E. W.	1928.	*J. Exp. Med.,* xlviii, 493.
,, ...	1938.	This *Journal,* xlvii, 423.

616 . 13—002—031 . 63—021 . 6

UNSUCCESSFUL ATTEMPTS TO PRODUCE PERIARTERITIS NODOSA EXPERIMENTALLY

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and

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During the last five years clinical and histological evidence has been given that periarteritis (polyarteritis) nodosa may be caused by sensitisation to drugs or foreign serum. Rich (1942 *a* and *b*, 1945) has reported nine patients who, after treatment with foreign serum or a sulphonamide drug or both, or with iodine, showed signs of sensitisation shortly before death and periarteritis nodosa at autopsy. Others have recorded similar findings after treatment with sulphonamides or arsenic (Black-Schaffer, 1945 ; Miller and Nelson, 1945 ; Perilman, 1946), and Bergstrand (1946) has described periarteritis nodosa as one of the lesions found in four patients who, before death, had shown a variety of allergic symptoms such as asthma, transient lung infiltration, blood eosinophilia, nasal polypi and abdominal pain with diarrhœa.

In addition, Rich and Gregory (1943 *a* and *b*) have reported that they produced periarteritis nodosa and lesions resembling rheumatic carditis in rabbits by intravenous injections of horse serum. Their method was based on that of Fleisher and Jones (1931), who studied the clinical manifestations of serum sickness in rabbits. In a letter written in June 1946, Professor Rich gave us the following summary of the results which he and Dr J. E. Gregory have obtained :—" Looking over the organs of 45 rabbits treated with horse serum, 20 show necrotising inflammatory arterial lesions, 6 inflammatory only and 19 no lesions at all. However, I remember a series of 10 rabbits of which only one showed necrotising lesions. I may repeat that we have never observed the necrotising lesions in control rabbits maintained in the laboratory in the same conditions at the same time ". Professor Rich wrote further :—" It is our belief that the irregularity with which periarteritis nodosa develops in rabbits during serum sickness is due to individual differences in the rabbits rather than to differences in the serum, for some of a series injected in the same manner with the same serum develop the lesions and some do not, and this regardless of the development of hypersensitivity ".

In order to emphasise that these very interesting arterial lesions cannot yet be produced at will, this report is made of unsuccessful attempts to produce periarteritis nodosa in four groups of rabbits.

In one experiment, the method outlined by Rich and Gregory (1943*a*) was used. Sterile filtered horse serum without preservative, kindly supplied by Dr L. F. Hewitt, was administered to 22 rabbits. Of these, 16 in three groups were adult males of mixed colours and breeds ; a fourth group consisted of six adult male albinos. The programme of injections of each rabbit was as follows :—

" X " day.   10 c.c. of horse serum per kg. body weight intravenously.
" X " day+11.   0·1 c.c. horse serum intracutaneously as test of skin sensitivity.

" X " day+17.   1·0 c c horse serum intravenously.
" X " day+19.   10 c. c. horse serum per kg. intravenously.
" X " day+26.   0 1 c. c. horse serum intracutaneously.
" X " day+33.   2 c c horse serum intravenously (in some animals only).
" X " day+28 35.   Killed by air embolism.

Of the 22 rabbits, 14 showed greater local reactions to the intracutaneous injections of serum after the first intravenous injection than they did before it , 8 did not show this evidence of increased sensitivity ; 2 animals showed severe distress soon after the second or later intravenous injections and several showed mild distress, but none died from this cause.

A careful naked eye examination was made of each animal immediately after death : periarteritis nodosa was not found in any. From 15 rabbits of this experiment (including at least 2 in each of the four groups), the myocardium, kidneys, liver, spleen and lungs were examined microscopically by Drs A B. Bratton, S H. G. Robinson and C. C. Bryson, who reported that no lesion recognisable as periarteritis nodosa was found in any section. In a few instances " chronic inflammatory infiltration " and " one focus of infiltration in relation to an arteriole but not affecting its wall " were found in the myocardium, but these trivial lesions were almost matched by similar ones in three of six rabbits which had not been injected with serum at any time Such lesions have been described by Miller (1924) as spontaneous interstitial myocarditis of rabbits.

In a second experiment, 10 male rabbits weighing from 1 6 to 2·1 kg. of mixed breeds were used. Seven were mixed colours and three albinos Wellcome brand " Horse Serum " no. 2 (Burroughs Wellcome) was given as follows .—

" X " day   Intravenous injection of 10 c c. per kg. body weight of horse
                     serum to all the rabbits
" X "+12 days   Intradermal injection of 0 1 c. c. horse serum for skin test.
" X "+17   „   Intravenous injection of 1 c c. of horse serum given very slowly.
" X "+19   „   Seven rabbits given 20 c.c. per kg. body weight of horse
                     serum intravenously.
" X "+21   „   Intradermal injection to remaining 3 rabbits of 0 1 c c. serum,
                     for skin test. These rabbits were killed 3 days later with
                     chloroform
" X "+26   „   Intradermal injection of 0 2 c c. serum for skin test
" X "+28   „   Four rabbits killed. The remaining three rabbits received
                     10 c c. per kg. body weight of serum intravenously.
" X "+33   „   Intradermal injection of 0 2 c.c serum for skin test
" X "+35   „   All animals killed by chloroform.

During the course of the injections, rectal temperatures were taken daily and careful observations were made for signs of cutaneous flushing, especially in the ears The tissues were fixed in formol alcohol and sections were stained with Ehrlich's acid hæmatoxylin and eosin and van Gieson's stain.

In this second experiment, a transient rise of 103-104 2° C. was observed between the 5th and 8th day after the first injection in all rabbits No definite sign of cutaneous flushing was seen. The reaction to the first skin test was only a faint erythema 5-10 mm. in diameter. The later tests showed more active response, with necrosis and hæmorrhage in the erythematous swellings of 25-35 mm. in diameter.

In the second experiment, the following organs were examined micro scopically, namely in all 10 rabbits, heart, lungs, liver, kidneys, spleen, pancreas, stomach, intestine, colon, testis, adrenal, brain, muscle of abdominal wall and extensor muscles of thigh, aorta, carotid and femoral arteries, axillary and mesenteric lymph glands and subcutaneous tissue of abdominal wall Only a few mild lesions were found, namely slight evidence of the spontaneous

logical study of these tumours will usually reveal the presence of neoplastic round cells and epithelial cell groups. Tumours of the kidney composed wholly of striated muscle, however, have not been described hitherto.

Histogenesis. The histogenesis of these tumours is satisfactorily explained only on embryological grounds. The histogenesis of the Wilms tumour depends chiefly on the period of embryonic life at which the tumour anlage develops. The renal rhabdomyoma may be interpreted as arising from the mesoderm before it has differentiated into nephrotome and lateral plates. The cells of this primitive germinal tissue are multipotent, and constitute the common precursor of both myotome and nephrotome. In the present instance the cells which separated to form the future tumour anlage have differentiated in one direction only—as muscle cells, many of which are striated.

Naked-eye appearance. The renal rhabdomyoma here recorded presents several characteristic features. It is of a large size and sharply demarcated from the renal tissue, which it displaces and distorts. It is solid and opaque, and the cut surface shows whorled markings which resemble those seen in uterine myomata.

Microscopic examination. No kidney tumour can be regarded as adequately investigated unless microscopic sections are taken from many different parts, since different portions of the same tumour may present different histological features. Especially is this so in the Wilms tumour, where, although the appearance may be that of an embryonal sarcoma, careful search will usually reveal epithelial elements. For this reason an extensive histological examination of the present specimen was carried out, and it has been shown that the tumour consists of one type of tissue only ; no epithelial or other complexes have been found. It is composed of interlacing bundles of well-formed fibres, morphologically and tinctorially resembling muscle. Many of these cells are well differentiated and show both longitudinal and cross striation. The cells resembling smooth-muscle elements are voluntary muscle fibres devoid of cross striation, and all gradations between these two forms and more primitive cells are encountered.

Many of these cells resemble the early stages of ontogenic development of striated muscle (Jordan and Kindred, 1937 ; Maximow and Bloom, 1938). Cappell and Montgomery (1937) believe that the malignancy of rhabdomyomata depends on these primitive elements, which are liable to metastasise and in their new positions may continue to grow and to differentiate to some extent.

Mode of growth. The course of these growths is rapidly progressive. The large size of the present tumour is a conspicuous feature, and this volume was attained in a relatively short time. The renal tissue is undergoing atrophic changes, being compressed and stretched peripherally by the tumour, from which it is separated by a narrow zone of connective tissue. There is no evidence of invasion of vessels. The presence of regressive changes is in part the result of activity of the growth, in part, no doubt, the effect of deep X-ray therapy.

Death was presumably due to renal failure from pressure destruction of the kidneys and not to true malignancy.

Summary

1. A case of bilateral renal rhabdomyoma in an infant is described. There is no previous record of a pure rhabdomyoma of the kidney in the literature.

2. Many sections taken from various parts of one of the tumours show only one type of tissue. The presence of cells showing both longitudinal and cross

PLATE LXXI

RHABDOMYOMA OF KIDNEY
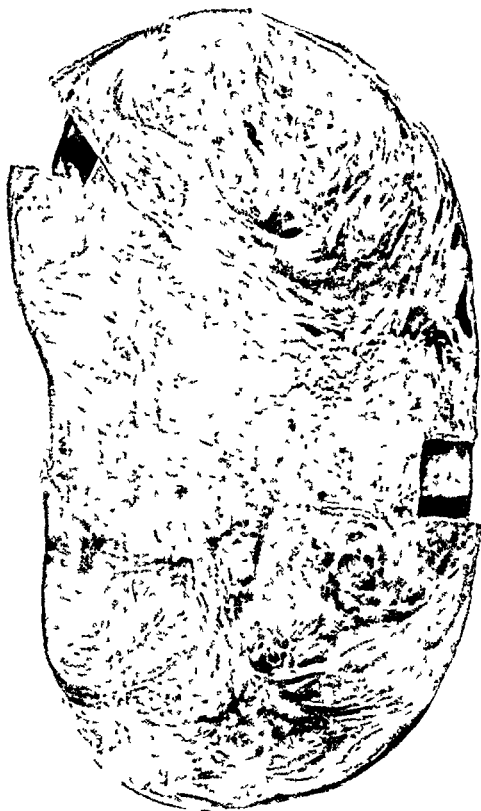
FIG 1 —Cut surface of one of the tumours showing whorled structure   Natural size

RHABDOMYOMA OF KIDNEY
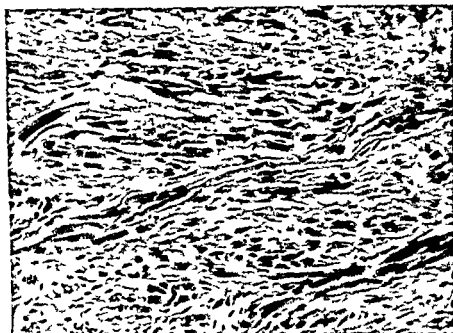
FIG 2.—Portion of tumour showing well-formed muscle fibres ×80

FIG 3.—Muscle fibres showing well developed cross striation ×210
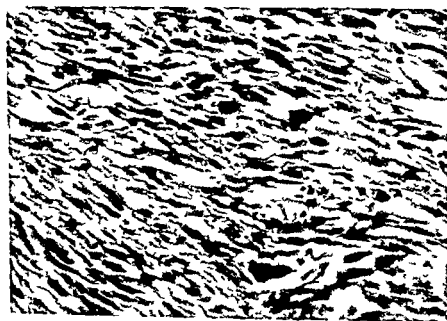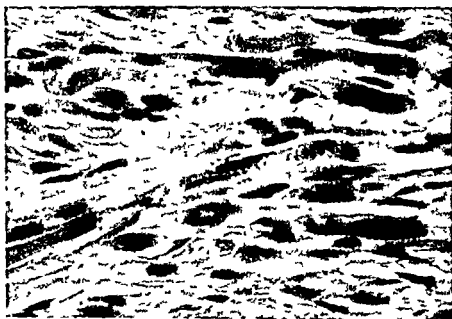
FIG 4.—A more cellular portion of the tumour showing cells of immature form ×120

striation and the absence of other elements indicate that the tumour is one of striated muscle.

3. From their morphology and staining reactions, it is concluded that the elongated, strongly acidophil cells which constitute a large part of the tumour are myoblastic in nature.

4. The histogenesis of this tumour is explained on embryological grounds. All stages in the development of the muscle cells are found.

REFERENCES

CAPPELL, D. F., AND MONTGOMERY, G. L.	1937.	This *Journal*, xliv, 517.
EWING, J.	1940.	*Neoplastic diseases*, 4th ed., *Philadelphia* and *London*, p. 243.
JORDAN, H. E., AND KINDRED, J. E.	1937.	*A textbook of embryology*, 3rd ed., *New York* and *London*, p. 483.
LIEBERTHAL, F.	1931.	*Surg. Gyn. Obst.*, liii, 77.
MCCURDY, G. A.	1934.	This *Journal*, xxxix, 623.
MAXIMOW, A. A., AND BLOOM, W.	1938.	*A textbook of histology*, 3rd ed., *Philadelphia* and *London*, p. 157.

A LIQUID MEDIUM BUFFERED AT pH 9·6 FOR THE DIFFERENTIATION OF *STREPTOCOCCUS FÆCALIS* FROM *STREPTOCOCCUS LACTIS*

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Streptococcus lactis and *Streptococcus fæcalis* are now established as distinct species, but there still appears to be difficulty in distinguishing between them. Serologically *Str. fæcalis* falls into group D (Sherman, 1938; Graham and Bartley, 1939), *Str. lactis* into group N (Shattock and Mattick, 1943-44), and they may be readily distinguished by culture in appropriate media. For this purpose Sherman and Stark (1934) suggested four " tolerance tests ": growth at 45° C., survival for 30 minutes at 65° C. in sterile skim milk, growth in lactose nutrient agar at pH 9·6, and growth in lactose nutrient agar containing 6·5 per cent. NaCl. These tests did not receive the attention they deserved, possibly because they were inadequately defined in the original paper. In some quarters they have fallen into disrepute, no doubt because of a lack of uniformity in technique and because some workers have attributed to all members of group D characteristics applicable only to *Str. fæcalis* and its variants, thereby concluding erroneously that these tests have little value as differential criteria.

Shattock and Mattick found that without exception *Str. fæcalis* would grow at 45° C. and at pH 9·6 (using a liquid medium), while *Str. lactis* failed to do so. On the other hand they found while *Str. lactis* failed to survive for 30 minutes at 65° C. in skim milk, many strains of *Str. fæcalis* also were unable to withstand this treatment, though reduction of the temperature to 60° C. and substitution of dextrose Lemco broth for skim milk were useful modifications.

Incubation at 45° C. in a thermostatically controlled water-bath presents little difficulty, but maintenance of so high a pH as 9·6 in a medium may be troublesome, although Sherman and Stark made no reference to the precautions

necessary. Reproducible results have been obtained in this laboratory by incubating dextrose Lemco broth cultures in an anaerobic jar containing soda-lime (Shattock and Mattick); but even with these precautions the fall in pH during handling and subsequent incubation was not easy to control. There is apparent difficulty in reproducing experimental conditions, particularly in different laboratories, and to provide a more satisfactory technique a liquid medium buffered at pH 9·6 has been devised.

Preparation of medium

Components.

I. Dextrose Lemco broth.

Dextrose 10 g., Lab Lemco 10 g., peptone (Evans) 10 g., NaCl 5 g., tap water 1 litre. pH 7·0. Autoclave at 15 lb. for 20 minutes.

II. Buffer solution (Clark, 1928). Glycocoll 7·505 g., NaCl 5·85 g., freshly boiled glass-distilled water 1 litre. This solution also may be autoclaved and stored in a well-stoppered flask. Mix 6 parts with 4 parts of N/10 NaOH by volume.

To 900 ml. of dextrose Lemco broth add 100 ml. of the buffer solution and adjust to pH 9·8 with N NaOH. Store overnight in a stoppered flask in the cold to complete precipitation. Filter through a Seitz filter and immediately distribute with sterile precautions into ¼-oz. McCartney bottles, leaving the minimum of air space at the top. This medium should be used within 48 hours. It is essential that the pH of uninoculated control bottles should be checked electrometrically (glass electrode) immediately before and after incubation. The initial pH should be $9·60 \pm 0·02$ and should not drop during incubation by more than 0·04 units. Care must be taken to ensure that the temperature of the medium is approximately 18° C. when checking the pH on the glass electrode.

Technique of test

The medium is inoculated with 2 loopfuls (4 mm. diam.) of an 18-hour dextrose Lemco broth culture, incubated at 30° C. $\pm 1·0$° C. for 24 hours and inspected for growth. Control bottles of dextrose Lemco broth+buffer (I and II above) adjusted with N HCl to pH 7·0 should be inoculated with each strain examined. Known positive and negative controls are included in each test.

To test the efficacy of this medium for the differentiation of *Str. fœcalis* from *Str. lactis*, 34 strains of *Str. fœcalis* and variants and 18 strains of *Str. lactis* were examined. A further 30 cultures, all shown by serological methods to belong to group D but comprising strains other than *Str. fœcalis*, were included.

The group-D cultures used in this investigation had been isolated from a variety of sources : human and bovine fæces, dried milk, pasteurised milk, sheep's-milk cheese, urine, and cases of endocarditis ; 5 cultures from the National Collection of Type Cultures were also included.

The cultures of *Str. lactis* had been isolated from milk or from starters used for cheese-making. *Str. cremoris*, which also belongs to group N, was not included because its differentiation from *Str. fœcalis* or other group-D streptococci presents no difficulties.

All cultures were grouped serologically and were further subdivided by biochemical and cultural tests ; where possible they were identified as accepted species and variants within groups D and N (Shattock and Mattick, 1943-44 ; Shattock, 1944, 1945). The table shows the species and variants growing at pH 9·6.

Results

Str. fœcalis, Str. fœcalis var. *liquefaciens* and *Str. fœcalis* var. *zymogenes* and their variants (a total of 34 strains) all grew vigorously at pH 9·6, whereas the

18 strains of *Str. lactis* and variants consistently failed to grow, thus confirming Sherman and Stark's claim that growth at pH 9·6 may be used as a differential criterion for *Str. fæcalis*.

TABLE

Growth of group-D and group-N streptococci in a differential medium buffered at pH 9·6

Organism	No. tested	No. growing at pH 9 6
Group D		
Str. fæcalis and variants	22	22
Str. fæcalis var. *liquefaciens*	3	3
Str. fæcalis var. *zymogenes* and variants .	9	9
Str. durans and variants	11	9
Str. bovis	9	0
Unclassified	10	0
Group N		
Str. lactis and variants	18	0

Str. durans gave less consistent results because only 9 of 11 strains grow under the conditions of these experiments. The failure of some strains of *Str. durans* to grow at pH 9·6 is recognised (Smith and Sherman, 1938; Shattock, 1945).

The 9 strains of *Str. bovis* all failed to grow and this is again in accordance with the findings of Sherman (1937) and Shattock and Mattick (1943-44).

The remaining 10 group-D strains listed as "unclassified" failed to grow. These cultures were shown serologically to belong to group D but they differed from *Str. fæcalis* in being less resistant to heat. Eight of the 10 had little or no reducing power and 5 did not grow readily at 45° C. While they approximated most closely to *Str. bovis*, they failed to ferment raffinose, which is regarded as an important characteristic of that species.

Summary

1. A liquid medium buffered at pH 9·6 has been devised primarily for the differentiation of *Str. fæcalis* from *Str. lactis*.

2. The ability of various species within group D to grow in this medium has been determined.

Our thanks are due to Mr L. G. Newland for technical assistance.

REFERENCES

CLARK, W. M.	. . . . . .	1928.	The determination of hydrogen ions, 3rd ed., *London*, p. 206.
GRAHAM, N. C., AND BARTLEY, E. O.		1939.	*J. Hyg.*, Camb., xxxix, 538.
SHATTOCK, P. M. F.	. . . .	1944.	*Proc. Soc. Agric. Bact.*, 69.
"	. . . .	1945.	*Proc. Soc. Appl. Bact.*, 18.
SHATTOCK, P. M. F., AND MATTICK, A. T. R.		1943-44.	*J. Hyg.*, Camb., xliii, 173.
SHERMAN, J. M.	. . . .	1937.	*Bact. Rev.*, i, 3.
"	. . . .	1938.	*J. Bact.*, xxxv, 81.
SHERMAN, J. M., AND STARK, P.	. .	1934.	*J. Dairy Sci.*, xvii, 525.
SMITH, F. R., AND SHERMAN, J. M.		1938.	*J. Inf. Dis.*, lxii, 186.

616 . 155 . 1—076 . 5 : 612 . 415 (Cuniculus)

RETICULOCYTOSIS IN SPLENECTOMISED RABBITS

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There is need for a method of assay of the anti-anæmic factor of liver extracts other than by testing their effect in known cases of pernicious anæmia. The use of splenectomised rabbits for this purpose has been suggested by Jacobson and Williams (1945 a and b), who found that such animals show a reticulocytosis on the fourth or fifth day after injection with potent liver extracts. These findings have received some confirmation recently from Bavin and Middleton (1946). Further experiments with splenectomised rabbits have been carried out here, and some incidental observations made on the counting of reticulocytes.

Experimental procedure

The rabbits were does weighing from 2·5 to 3·5 kg. They were splenectomised under Nembutal anæsthesia and the post-operative reticulocytosis allowed to settle. Each extract was given to groups of 2-6 animals. Some of the extracts were also tested on patients with pernicious anæmia. All the substances tested were injected intravenously, daily reticulocyte counts being made for the six days before and after the injection.

Results

Consideration of the counts over the 12-day periods showed considerable variation from day to day and in different animals. In order to determine what part of the variation could be ascribed to the injection, the counts were re-tabled to show the differences between the first and fourth, second and fifth, and third and sixth days of the period before injection, and the three corresponding differences after the injection. A positive result would then be shown by an increase in the differences after injection. To test this, an analysis of variance was set up for each group of tests, the variance being separated into components derived from rabbit differences, day differences, the injection and interactions. The significance of the injection component was tested by comparing the mean square derived from it with that from the residual, using the tables of variance ratios given by Mather (1943). This method would not detect an increase of reticulocytes in the first three days after injection, but comparison of the figures for these days with those preceding injection did not show any such increase.

Table I gives these steps for the figures obtained from five animals tested with " Cambridge " extract. The results from all the experiments are summarised in table II. Consideration of the counts on the basis of a binomial distribution, as discussed by Jacobson and Williams (1945a), in which a rise above 3·5 per cent. is held to be significant, leads to the same conclusions as with our method of analysis.

Several types of preparation were used, and where the figure for the effective dose in man is given in table II it is the amount observed to produce a maximal response over a period of 14 days in a patient with pernicious anæmia. Extract 303 was a special fraction supplied by Glaxo Laboratories Ltd. and known to be active in a dose of 27 mg. It was a dry powder free from preservative.

Extract 930, also an experimental batch, was supplied by British Drug Houses Ltd   This extract had been tested clinically, and though active the response to doses of 100 mg was submaximal   Extracts B329, B342 and B345 were different batches of Anahæmin supplied for clinical testing by British Drug Houses Ltd   These were all tested clinically and found to give maximal

TABLE I

Daily reticulocyte counts of 5 rabbits for 12 days, injected with "Cambridge" liver extract after the count on the sixth day   The figures are the reticulocytes noted in counts of 2000 red cells

Day	1	2	3	4	5	6	7	8	9	10	11	12
Rabbit 1	33	29	40	31	33	41	25	32	31	76	64	48
,, 2	58	74	54	52	54	46	52	48	62	76	104	94
,, 3	56	56	66	70	46	42	46	38	52	52	70	100
,, 4	28	19	32	34	33	32	54	66	54	104	84	102
,, 5	28	46	48	48	40	38	38	42	50	56	88	64

Results expressed as differences between the counts on the first and fourth days etc , giving three such differences before injection for comparison with three differences after injection   The differences are seen to be increased after injection

Day	4 1	5 2	6 3	10 7	11 8	12 9
Rabbit 1	−2	4	1	51	32	17
,, 2	−6	−20	−8	24	56	32
,, 3	14	−10	−24	6	32	48
,, 4	6	14	0	50	18	48
,, 5	20	−6	−10	18	46	14

Analysis of variance derived from the table of differences

Class	Sum of squares	Degree of freedom	Mean square
Rabbits	131	4	33
Days	217	2	108
Injection	8,979	1	8979
Injection day interaction	469	2	235
Injection rabbit interaction	836	4	209
Residual	4,100	16	256
Total	14,732	29	

$$F = \frac{\sigma_1^2}{\sigma_2^2} = \frac{8979}{256} = 35$$

This indicates a significant increase after injection, as such a value would be obtained by chance less than once in a thousand times

responses over 14 days in the doses shown in the table   "Cambridge" extract was "Hepastab Forte", its clinical activity was not examined   "Examen" was ordinary commercial extract   Folic acid was dissolved for injection in buffered phosphate solution at pH 8 0, pterin in isotonic sodium bicarbonate. Of the preparations examined only one ("Cambridge") gave a significant

response; with it, peaks of 3·8 per cent. to 5 per cent. were obtained from four to six days after injection.

TABLE II

The preparations examined, with the doses given and the reticulocyte response obtained

Extract	Effective dose in man	Test dose in rabbit	No. of animals	Result
Intact animals				
303	27 mg.	27 mg.	6	Negative
Splenectomised animals				
303	27 mg.	27 mg.	6	Negative
930	...	100 ,,	6	,,
B329	130 mg.	130 ,,	3	,,
B342	130 ,,	195 ,,	2	,,
B345	80 ,,	160 ,,	2	,,
Cambridge	...	140 ,,	5	Significant reticulocytosis
Examen	...	1 ml.	3	Negative
Pterin	...	10 mg.	2	,,
Folic acid	...	25 ,,	4	,,

Reticulocyte counting. A wet method was used, blood being mixed with stain, spread under a coverslip, sealed with paraffin and allowed to settle. Two films were made for each count and 1000 red cells counted from each in groups of 200, from the centre and from each corner. Marcussen (1938-39) showed that wet preparations give a fortuitous distribution of reticulocytes among the other red cells. In order to check this distribution and to trace the effect of making counts from the centre or corners of the film, a further analysis of variance was set up based on 96 pairs of counts. This indicated that counts made from the centre are significantly lower than those from the corners. The difference is not great, the means being 1·67 and 2·00 per cent. respectively. More consistent results would not have been obtained by taking all counts from the centres. There was no significant variation in the pairs of counts made on each occasion, nor in the counts from different corners. These results suggest that while the distribution of reticulocytes in a wet preparation is not completely fortuitous, accurate results will be had if the method of counting is consistent.

Discussion

While it seems to be established that, given the right conditions, some liver extracts will produce a reticulocytosis in the splenectomised rabbit, it is evident that when administered in comparable doses, not all potent extracts have this power. Extracts 329 and 342, for example, when tested clinically in doses of 130 mg., gave reticulocyte responses of 23 and 20 per cent. respectively, but they gave no response in the rabbit in doses of 130 and 195 mg. There are several possible explanations for these discrepancies. Jacobson and Williams (1945a) point out that the presence of antiseptic in the extract as supplied for clinical use may inhibit the response; one of their preparations gave no response with a large dose containing tricresol, whereas a smaller dose with less or no tricresol was active. Of our preparations extract 303 and folic acid were free from antiseptic but gave no response in the rabbit test. Bavin and

Middleton mention that some animals did not respond to extracts which were active in others. These are questions of technique, and apart from them it is becoming evident that there are a number of substances which have a curative effect in pernicious anæmia and which are not identical; folic acid for example is not present in some active extracts. Liver extracts produced by different methods probably differ in their active principles, and it may well be that some of these will not produce a response in the splenectomised rabbit. In its present form, therefore, this test does not seem to be of practical value in the assay of liver extracts.

Summary

Normal and splenectomised rabbits have been injected with liver extracts and other substances and the reticulocyte response noted.

One liver extract was found to produce a significant reticulocytosis but other potent extracts and folic acid were without effect.

The distribution of reticulocytes in wet preparations is not fortuitous, but accurate results will be obtained if the method of counting is consistent.

We wish to thank Mr D. G. Champernowne for help with the statistical matter in this paper, and Professor L. J. Witts for advice in its preparation.

REFERENCES

BAVIN, E. M., AND MIDDLETON, 1946. *Nature*, clviii, 627.
T. R.
JACOBSON, W., AND WILLIAMS, 1945a. This *Journal*, lvii, 101.
S. M.
  „   „   „   „   1945b. This *Journal*, lvii, 423.
MARCUSSEN, P. V. . . . . . . 1938-39. *Folia Hæmatol.*, lxi, 49.
MATHER, K. . . . . 1943. Statistical analysis in biology,
London.

THE ALLERGIC REACTION OF THE KIDNEY TO SULPHONAMIDE MEDICATION

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Following the universal use of the sulphonamide compounds there soon came reports of serious complications. Some of the earliest and most serious of these are the blood dyscrasias, especially agranulocytosis and the hæmolytic anæmias. Renal damage is another complication which was early reported and has become an increasingly common result of sulphonamide treatment. The clinical symptoms of renal impairment in all these types have been more or less identical in fatal cases, being characterised by oliguria, anuria, hyper-azotæmia and uræmia. Histologically the renal lesions have been rather varied. Some of the more frequently encountered are well known; others, more lately reported and of less frequent occurrence, are less familiar.

The histological lesions so far reported may conveniently be classified into several different types. Possibly the most common and best known is the

response; with it, peaks of 3·8 per cent. to 5 per cent. were obtained from four to six days after injection.

TABLE II

The preparations examined, with the doses given and the reticulocyte response obtained

Extract	Effective dose in man	Test dose in rabbit	No. of animals	Result
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B342	130 ,,	195 ,,	2	,,
B345	80 ,,	160 ,,	2	,,
Cambridge	...	140 ,,	5	Significant reticulocytosis
Examen	...	1 ml.	3	Negative
Pterin	...	10 mg.	2	,,
Folic acid	...	25 ,,	4	,,

Reticulocyte counting. A wet method was used, blood being mixed with stain, spread under a coverslip, sealed with paraffin and allowed to settle. Two films were made for each count and 1000 red cells counted from each in groups of 200, from the centre and from each corner. Marcussen (1938-39) showed that wet preparations give a fortuitous distribution of reticulocytes among the other red cells. In order to check this distribution and to trace the effect of making counts from the centre or corners of the film, a further analysis of variance was set up based on 96 pairs of counts. This indicated that counts made from the centre are significantly lower than those from the corners. The difference is not great, the means being 1·67 and 2·00 per cent. respectively. More consistent results would not have been obtained by taking all counts from the centres. There was no significant variation in the pairs of counts made on each occasion, nor in the counts from different corners. These results suggest that while the distribution of reticulocytes in a wet preparation is not completely fortuitous, accurate results will be had if the method of counting is consistent.

Discussion

While it seems to be established that, given the right conditions, some liver extracts will produce a reticulocytosis in the splenectomised rabbit, it is evident that when administered in comparable doses, not all potent extracts have this power. Extracts 329 and 342, for example, when tested clinically in doses of 130 mg., gave reticulocyte responses of 23 and 20 per cent. respectively, but they gave no response in the rabbit in doses of 130 and 195 mg. There are several possible explanations for these discrepancies. Jacobson and Williams (1945a) point out that the presence of antiseptic in the extract as supplied for clinical use may inhibit the response; one of their preparations gave no response with a large dose containing tricresol, whereas a smaller dose with less or no tricresol was active. Of our preparations extract 303 and folic acid were free from antiseptic but gave no response in the rabbit test. Bavin and

boundary was poorly defined, and both cortex and medulla showed small hæmorrhagic areas and greyish stripes like streaks of pus. In the calyces and pelves were neither crystals nor concretions.

Microscopic examination. The glomeruli did not show any marked alteration except hypertrophy and swelling of the parietal cells of Bowman's capsule in a few glomeruli. The tubular epithelium showed moderate clouding of the cytoplasm, but there was no obvious degeneration or necrosis. No sulphonamide crystals could be found in the collecting tubules. The blood vessels were greatly distended with blood and a number of them were surrounded by small areas of hæmorrhage. The small muscular arteries showed distinct focal fibrinoid degeneration of the entire wall, in some cases leading to necrosis and subsequent rupture. The interstitial tissue was œdematous and contained a large number of plasma cells, some lymphocytes and a number of eosinophil cells, but very few polymorphs. The cellular infiltration was mainly localised around the blood vessels, but was also found diffusely between the parenchymatous elements. Around the ruptured vessels the cellular infiltration was so abundant as to resemble small abscesses. In Gram-stained preparations no bacteria were found.

Discussion

It is obvious from the description that the renal lesion presents a very exceptional and striking picture. The kidneys show acute degeneration of the walls of the smaller arteries and an inflammatory reaction dominated by the presence of plasma cells and eosinophils. The features are similar to those found in acute allergic reactions.

The important question is, has the kidney lesion described been caused by the primary disease of the patient, namely a subacute hidradenitis, or has it been caused by the treatment? With our present knowledge, it is difficult to explain the renal lesion as the result of an ordinary hidradenitis. The bacteria usually involved in such lesions may be active for prolonged periods, causing many relapses without the occurrence of renal changes of the kind described. The most probable renal complication in such a case would be an embolic focal nephritis, or possibly a chronic diffuse nephritis.

The sulphonamides, on the other hand, are known to cause allergic reactions, and in the present instance a reasonable time had lapsed between the first and second periods of medication to allow of allergic manifestations making their appearance. The initial morbilliform rash and the œdema of the face are both well known types of allergic reaction and are rather common in connection with the sulphonamide drugs. It is therefore not unreasonable to suggest that the renal lesion represents a new and fatal sequence in an allergic reaction to the sulphonamides.

Another question is the correlation between the anatomical findings and the symptoms—oliguria, anuria, hyperazotæmia and uræmia. Most authors stress the importance of œdema and the resulting increased pressure as a cause of the impaired functional activity of the kidney. Peters (1945-46) claims to have proved this experimentally with an artificial nephron.

In the present case there was no mechanical obstruction from sulphonamide crystals and the œdema was not severe enough to cause obstruction to the blood flow through the smaller vessels of the kidney. On the contrary, the vessels were widely distended, giving the picture of an intense hyperæmia. Such a picture, however, does not necessarily indicate a good blood flow. The hyperæmia as described, combined with multiple capillary hæmorrhages, points to the existence in this case of the circulatory conditions of pre-stasis and stasis, characterised by distended vessels and a marked increase of their permeability, slow blood flow, and finally complete blocking of the vessels by blood cells, with absence of plasma. The presence of such a circulatory disorder—a common

feature of the allergic reaction—may explain the complete failure of urinary excretion.

In a discussion of the genesis of the renal failure and subsequent uræmia in these cases, a few words may be said about treatment. If the impaired renal function is the result of increased intrarenal pressure caused by renal œdema, then the correct treatment should be early decapsulation, before irreparable damage to renal tissue has developed. Peters especially has stressed this point and maintains that decapsulation is the only life-saving treatment. He advises that it should be performed as early as possible as an emergency operation, both in these cases and in others with a similar genesis, *e.g.* transfusion nephropathies and the crush injury kidney.

In all five different types of sulphonamide nephropathy, however, the clinical symptoms are similar, and possibly the clinical differentiation between the various anatomical types cannot yet be made except in cases of urolithiasis medicamentosa, provided the crystals are demonstrable in the urine.

A definite mode of treatment cannot therefore be proposed in cases like the one described, in which most probably the primary cause of the renal failure was the lesion of the vessels with subsequent stasis.

Patients given repeated treatment with sulphonamides, even in small doses, should be kept under close observation for early clinical symptoms of hyper-sensitivity, and particularly for simultaneous signs of renal impairment and diminished urinary output, in which case the administration of the drug should be immediately stopped.

Summary

Various types of renal lesion due to sulphonamide medication are mentioned. In the case reported, though the dose of the sulphonamide (sulphathiazole) was small, renal insufficiency developed with a fatal outcome. The history and the morphological lesion in the kidney indicate an allergic reaction to sulphathiazole. The importance of the vascular lesion and of stasis in the development of the renal failure in these cases is stressed. No definite mode of treatment can be proposed.

REFERENCES

BERGSTRAND, H. . . . . . . 1946. *Acta med. Scand.,* cxxiv, 309.
BLACK-SCHAFFER, B. . . . . 1945. *Arch. Path.,* xxxix, 301.
MORE, R. H., MCMILLAN, G. C., 1946. *Amer. J. Path.,* xxii, 703.
AND DUFF, G. L.
PETERS, J. T. . . . . 1945-46. *Acta med. Scand.,* cxxiii, 90.
WIRD, BLENDA . . . . . . 1942a. *Svensk Läkartidningen,* xxxix, 22.
       ,,   ., ... 1942b. *Ibid.,* xxxix, 124. (*Abstr.,* 1942, *Nordisk Medisin,* xvi, 2864.)

Addendum

Since this paper was written two other cases have come to autopsy which were very similar to the one here described. Another case showing acute glomerulitis has also been encountered. In these three cases sulphathiazole appeared to be the most likely causative agent.

<div align="right">576 . 851 . 49 (*Salm. singapore*)</div>

A NEW SALMONELLA SPECIES, *SALM. SINGAPORE*
(VI. VII; k←→e, n, x)

N. K. SEN

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In an investigation of salmonellosis in South-East Asia I encountered a new strain of Salmonella which was similar to *Salm. thompson* in many respects except in the non-specific phase (II-phase). In this phase the antigens were entirely different, being e, n, x instead of 1·5... of *Salm. thompson*. For this organism the name of *Salmonella singapore* is suggested because it was isolated in Singapore. The description and other details are presented below.

History of the bacterium

The organism was isolated from the stool of Otani, a Japanese private in Singapore. On 6.12.43 he suddenly developed a temperature of 101·8° F., with diarrhœa. He had pain in the abdomen and passed four loose motions on that day. Next day the temperature persisted and on the third day the fever subsided and the patient recovered.

General and biochemical characters

The colony on nutrient agar was 2-3 mm. in diameter, greyish white, semi-transparent and medium convex, with entire edge and smooth shining surface. In nutrient broth growth occurred with uniform turbidity. The organism was a Gram-negative motile bacillus 2·4 μ in length, with peritrichate flagella; it was non-sporing and non-capsulated. It fermented glucose, mannitol, dulcitol, sorbitol, inositol, maltose, arabinose, rhamnose, xylose, trehalose and dextrin, with production of acid and gas. Lactose, saccharose, salicin and adonitol were not fermented. A positive reaction was obtained with Bitter's solution and Simmons's medium containing arabinose, glucose, rhamnose and dulcitol respectively. Stern broth was positive in two days. Litmus whey became acid, later alkaline. Hydrogen sulphide was produced but no indol. Gelatin was not liquefied. The methyl-red test was positive while the Voges-Proskauer reaction and citrate-utilisation test were negative.

Serological characters

On serological examination it was found that this organism was agglutinated only by O sera of *Salm. thompson* and its variant *berlin* (VI. VII), *Salm. newport* var. *puerto rico* (VI. VIII) and *Salm. carrau* (VI. XIV. XXIV) and by the mono-specific serum from *Salm. thompson* var. *berlin* containing agglutinin to factor VII. These results suggested that the organism had the somatic complex VI. VII. Absorption of *Salm. thompson* (VI. VII) serum by the organism completely removed all agglutinins to *Salm. thompson*. Similarly, *Salm. thompson* removed all somatic agglutinins from a serum prepared against the new strain, thereby proving that the somatic complex was VI. VII.

In testing the flagellar antigens it was found that some colonies of this new organism were agglutinated to titre by *Salm. thompson* H (k) serum, other colonies which were agglutinated only slightly by this serum were agglutinated to titre by *Salm. abortus-equi* H serum, suggesting that this organism was

diphasic, the structure being represented by k←—→e, n, x. The H (e, n, x) phase was agglutinated by monophasic sera containing antibodies to factors n and x respectively. Absorption of *Salm. thompson* H (k) serum with the unknown organism H (k) completely removed all agglutinins to the homologous strain. Also absorption of *Salm. abortus-equi* serum with the unknown organism H (e, n, x) completely removed all agglutinins to the homologous strain. A serum prepared against the unknown organism agglutinated *Salm. thompson* H (k) and the unknown organism H (k) to the same titre, also this serum agglutinated *Salm. abortus-equi* H and the unknown strain H (e, n, x) to identical titres. Absorption of this serum with *Salm. thompson* H (k)+*Salm. abortus-equi* H removed all agglutinins to both phases of the homologous strain, thereby proving the H complex to be k←—→e, n, x.

Conclusions

A new species of Salmonella was encountered in South-East Asia. It was isolated from a case of enteritis and its antigenic structure appears to be VI. VII ; k←—→e, n, x.

Its e, n, x antigens (β-phase antigens) appear to be the same as those of *Salm. abortus-equi* (B. 202), but different from those of *S. chester*.

The name " *Salmonella singapore* " is suggested for this organism.

BOOKS RECEIVED

Recent advances in clinical pathology

General editor, S. C. DYKE. 1947. London: J. & A. Churchill Ltd.
Pp. xii and 468; 192 figs. on 34 plates and 22 text figs. 25s.

"The purpose of clinical pathology is to bring the laboratory into effective use in clinical medicine", says Dr Dyke in his preface. To this end the clinical pathologist must keep himself abreast of advances both in laboratory practice and in the general field of medicine. Perhaps no other speciality calls for so watchful an eye to be kept on the literature, and it is good news that Messrs Churchill's should have added this volume to their admirable "Recent advances" series. It is better news still that the book should be one so ably planned and edited, so instructively written and so well documented that its immediate reception is assured and its continued currency in perennial revisions almost inevitable.

The book is an outcome of various circumstances of the recent war; directly in that it is published under the auspices of the European Association of Clinical Pathologists, whose members, on the eve of the liberation of the Continent and with the prospect of the return of many of them to their homelands, wished to make a record of current practice in clinical pathology in this country; indirectly in that the huge widening of both hospital and public laboratory services under the E.M.S. and the concomitant recruitment of many of our own teachers and investigators into this branch of pathology provide both the market and the source of material for the book.

There are four sections of roughly equal bulk, though hæmatology sacrifices something to histology in the matter of pages and biochemistry to bacteriology in the number of chapters. The four sectional editors are R. Cruickshank (bacteriology), E. N. Allott (biochemistry), B. L. Della Vida (hæmatology) and A. H. T. Robb-Smith (histology). They and the general editor are to be congratulated both on their choice of subjects and on their selection of authors. Well over half the 40 contributors are members of our own Pathological Society; eleven are graduates of continental schools. The subjects of which they treat are all either of topical interest, *e.g.* anaerobic infections, photoelectric colorimeters, Rh factor and peripheral nerve biopsy, or recurring problems such as enteric infections, liver function tests, semen analysis and lymph-node biopsy, to which a new or more analytical approach can now be made in the light of recent knowledge or modern technique. In most of the chapters where space demands economy of exposition, emphasis is on technique rather than interpretation. Technical details are, in fact, lavishly given and the final chapter on routine histological technique is a veritable *vade mecum*. But as a whole the familiarity of the reader with standard laboratory procedures is assumed and the book is an appendix to and not a substitute for the ordinary bench manual. Thus the reader of chap. I on the laboratory diagnosis of enteric infections must have access to the Kauffman-White tables and must know their meaning. Incidentally he must also be able to recognise his old friends "aertrycke" and "Gaertner" under the names *Salm. typhi-murium* and *Salm. enteritidis*. Again none but the already experienced histologist can aspire to confident diagnosis by way

of aspiration biopsy (chap. XXXII). A few of the topics such as typhus fever, cell counts in serial tumour biopsies and the wet film technique in neurosurgery are clearly of limited application unless the particular location of the laboratory calls for a practical interest in these matters, but most of the other subjects are the almost daily concern of any busy general laboratory.

All of us must have found on occasion, when faced with a problem outside our own particular experience or training, that neither native wit nor the closest study of our text-books proves so helpful as a visit to one who has specialised in the subject to talk with him, to hear his critical comments and to watch his technique. Back in our own departments we miraculously find our own confidence restored, our vision clear and the necessary procedures practicable. The best chapters of this book have just the quality of that half-hour's benchside talk with the expert. Herein lies its especial value to that ever-growing body of general pathologists who have to work more or less in isolation and who are keen to use the best methods of approach to their problems so that they reach the best answers. But the expert or specialist himself will hardly fail to profit by reading it. He may be, indeed he should be, familiar with the papers and procedures quoted in the sections on his own speciality, but it is unlikely that he will take great exception to any of the views expressed. He is more likely to be conscious of what has been left out. This, however, is not to be taken as a wholesale adverse criticism, for it is certainly a very great achievement to have gathered so much up-to-date information in a single volume, and by limiting each chapter to some ten or a dozen pages the review of each subject is made easily readable in a single session.

Recent literature is fully covered and each chapter ends with a list of references. More than half of these 636 references are to papers and books published since 1939 and only some 8 per cent. antedate 1930 (including a tabulated reference to Billroth, dated 1858, which is surely a record entry for a volume in the " Recent Advances " series !). The index is adequate, though it may be of less use than the table of contents, which shows the placing of matter so clearly. Most of the essays are well written but lapses in style are sometimes encountered. The most readable chapters are generally those which come from the pens of authors who have already written a good deal on their subjects, and style in writing is by no means the prerogative of those whose native tongue is English. Here and there style is impeded by the perpetuation or invention of ugly and unnecessary neologisms. These are usually of transatlantic origin and objection to their use in this country is based solely on grounds of æsthetic repulsion, bastard etymology and the fact that they are more often than not simply confusing alternatives to already familiar and explicit terms. Thus it comes as something of a shock to read in the otherwise excellent chapter XXXVII on the skin biopsy " basalioma " and " spinalioma " in the paragraph on epithelioma. One rather has a fancy for " pricklioma ", but this does not seem to have been thought of yet.

The majority of the 192 figures are photomicrographs in the section on histology, and 74 of these illustrate the chapter on aspiration biopsy, most of them at a magnification of 100 diameters. They tend to lose their effectiveness, partly from the low magnification employed (as also in those illustrating testicular biopsy and endometrial biopsy), partly from the very small size of the individual prints. Low-power photographs in particular suffer from reduction in area, as the feeling of a survey view is thereby lost, and when cell nuclei are reduced to the size of the grain of the plate not even a reading glass will help.

Finally, we sincerely hope that this volume will succeed to many

editions. It is planned in such a way that the editors' only difficulty will be in deciding which parts require revision, which can be dropped and what new chapters to add. A great service has been done to clinical pathology by this highly original enterprise and clinical pathologists the world over will readily subscribe to successive editions if the same quality, modernity and comprehensiveness can be maintained.

The pathology of traumatic injury

By JAMES V. WILSON. 1946. Edinburgh: E. & S. Livingstone. Pp. xi and 192; 61 text figs. (10 in colour). 20s.

This is very much a book which dates: it is a product of a local and temporarily intense interest in and concentration upon certain phenomena of common occurrence in war. Probably even now the period of its appeal is over and pathologists are again getting a more balanced perspective. Be this as it may, it is not suggested that the investigations urgently demanded *ad hoc* under the stimulus of war are unfruitful. Mostly knowledge advances in a series of jerks, and the spasmodic stimulus to the intensive study of shock, burns, traumatic anuria, fat embolism, blast injury and the wounds of war—which are the subjects treated of in the first part of the book—unquestionably has resulted in valuable gains in knowledge in all these fields. One effect—the publication of a book such as this—is probably ephemeral and as the centre of interest shifts so does the special emphasis decline, and as the new knowledge becomes absorbed into the general pathology of disease processes, so the specialised monograph goes out of print.

Dr Wilson's book was written " in the midst of a traumatic epidemic " and is based upon experience gained during service with the R.A.M.C. Its second part is devoted to a special consideration of injuries to the chest, the blood vessels, the abdominal organs, the nervous system, and the bones and joints. The field is a vast one for a book of less than 200 pages and the reader is left with the impression that the survey is perforce cursory. There is certainly a lack of personal doctrine in most of the subjects discussed, and whilst the divergent tenets of authorities are quoted the reader will look in vain for any critical analysis or judicial appraisement of these views. Many if not most of the illustrations are familiar and borrowed. It is to be hoped that other authors will not follow the irritating practice of acknowledging the source of these in the foreword rather than with the illustrations themselves.

There are numerous references which will be useful to those especially interested in the conditions described. There are also many minor errors such as Keinbock for Kienböck, Treuta for Trueta, " lewisites of phosgene ", " impregnateion " etc., and on p. 93 alone we find : " Andrew (for Alexander) Fleming ", " cocci-staphylococcus aureus and streptococcus pyogenic ", " Bacillus proteous " and " tetanus-gas gangrene " ; and the writer is rather cavalier in his treatment of tenses and numbers.

It is perhaps unfair to stress these errors, for the completion of such a book under field conditions must have been a very difficult task and one demanding both courage and enthusiasm.

Atlas of bacteriology

By R. CRANSTON LOW and T. C. DODDS. 1947. Edinburgh: E. & S. Livingstone. 166 figs. (167 in colour). 32s. 6d.

Those aware of the technical difficulties involved in accurate colour photography, especially of bacterial cultures, will agree that this atlas reaches a high standard in its reproductions of the common pathogenic

bacteria in films, cultures and histological sections. A number of protozoa, fungi and viruses are also illustrated. The atlas is intended for use with a standard text-book and the authors wisely allow their illustrations to speak for themselves. They do well to emphasise that " a rapid cursory glance will not reveal the details ". Indeed if the student overlooks that some of the cultures have been enlarged from 2 to 15 times he will gain an entirely false impression from looking at some of the pictures. The volume is well fitted to do for the medical student what its authors intend —to illustrate what he hears in lectures, sees in the practical class and reads in text-books. One possible disadvantage of a volume of this kind is not in the authors' power to overcome — namely, that students too readily accept the study of an atlas as a substitute for the trouble of making and examining as many original preparations as they might.

Die Immunitätsforschung, vol. I, Antikörper, part I

By R. DOERR. 1947. Vienna : Springer-Verlag. Pp. ix and 259; 19 text figs. Swiss Fr. 24.

The publications which may be grouped under the very comprehensive heading of " Immunity " are so numerous and so diverse that even the specialist will be glad to have available a concise and critical summary of the contributions to immunology which have been made during recent years.

In the first half of the first volume of a series of monographs which will cover the whole field of immunity, Professor Doerr has given a concise but readable account of recent work on antibodies, antigens and the serum reactions. English readers, who are well served by the monographs of Marrack and of Landsteiner and the immunological sections in Topley and Wilson's *Principles of bacteriology and immunity*, will find, inevitably, much that is familiar to them but will welcome summaries of the more important papers published during the last few years. Recent work on the nature and origin of antibodies has been summarised admirably and the value of this section of the book is enhanced by an account of the application of the electrophoresis method to the study of the characteristics of immune globulins, and of isotopes to the study of their origin. Other sections of the book contain useful accounts of the results obtained by the use of the electron microscope in the study of serological reactions and of recent advances in our knowledge of the agglutination of red corpuscles by the influenza and other strains of ultra-microscopical virus.

The summaries of individual papers and of groups of papers on allied topics are introduced by paragraphs which set out the present state of knowledge, the nature of the problems and the methods which have been used for their solution. Readers whose knowledge of physical chemistry may be slightly out of date may welcome an explanation of the application of this branch of science to the solution of serological problems. The critical and stimulating reviews with which the author has concluded the majority of the chapters are not the least valuable features of this book. Professor Doerr's account of the present state of our knowledge of antibodies will appeal to all who are interested as teachers and research workers in serology. If the other volumes are as good as this, the first to be published, the entire series should find a place in the library of every pathological laboratory.

Die Immunitätsforschung, vol. II, Das Komplement

By R. DOERR. 1947. Vienna : Springer-Verlag. Pp. vi and 74; 2 text figs. Swiss Fr. 7.50.

There can be few subjects in pathology about which so much has been written and so little is known as the substance in or function of fresh serum

which we call "Complement", and Professor Doerr has done well to include in a small volume of 74 pages summaries of so many papers published on this confused and perplexing subject. The book contains a short but sufficient account of recent achievements in splitting complement and of the distribution of the fractions or components of complement among the vertebrates. The very little which is known about the mode of action of complement is discussed, of the part it plays, with antibody, in cell death (in the case of certain bacteria), in cell destruction and in the escape of hæmoglobin from sensitised red corpuscles. The sections on the changes produced in some of the protozoa and on the neutralisation of viruses by antibody and complement help to provide a broader basis for the consideration of problems, the solution of which has been sought hitherto almost exclusively by the intensive study of hæmolysis. Complement is more than a reagent used in the Wassermann reaction. Professor Doerr's thoughtful summaries and his critical appraisement of the significance and relative value of the papers which he quotes add greatly to the value of this book, which is essentially a work of reference for the specialist in serology. But, as Professor Doerr has warned his readers, we still know very little, after more than forty years of intensive study, of the origin or functions of complement or of the part which it plays in health and disease, more particularly in the protection of the host against the parasite.

The microscope: its theory and applications

By J. H. WREDDEN. 1947. London: J. & A. Churchill. Pp. xxiv and 296; 272 text figs. and 38 figs. on 23 plates. 21s.

This book has the virtue of not being bookish. The author has apparently come to understand his subject by that uncommon route, the exercise of his own mind. Not that he has ignored the literature, indeed his quotations are extremely apt, but like the old-fashioned craftsman his knowledge is partly in his hand and so his explanations and descriptions have a freshness and clarity possessed by few books on the microscope. One feels he is neither a maker of lenses nor an academic transcriber: none the less to the academic reviewer the reference lists seem to be treated rather too casually—and split infinitives occur too often.

Apparently few of those who use the microscope for a living are expert in optical matters, and it must have been the uncomfortable experience of many pathologists and bacteriologists to be credited with a knowledge of optics which they could scarcely sustain. For those who have to help juniors and technicians this book will certainly prove valuable; it describes clearly the basic optical principles, the various parts of the microscope, both optical and mechanical, and the profitable ways of working. Most of the references are to Watson's products, although American and German apparatus is also discussed; but the author, like most English microscopists, is somewhat lacking in the utilitarianism which led to the Leitz IK objective and to the Zeiss pancratic condenser, neither of which is mentioned. Doubtless the present delays in book production explain the absence of reference to post-war English microscopes and the new phase microscope. The last forty pages—on the preparation of specimens—reveal the author's versatility, and may well stimulate other commercial laboratory workers to extend their use of the microscope, but the brief summary of histological methods, a subject with which the reviewer is familiar, is so dangerously superficial that he has his fears about the adequacy of some of the other techniques described. The historical introduction by Mr Watson-Baker might well have been longer and this last section cut to its gain.

A practical textbook of leprosy

By R. G. COCHRANE. 1947. London: Geoffrey Cumberlege, Oxford University Press. Pp. xi and 283; 183 text figs., frontispiece in colour. 42s.

Dr Cochrane's reputation and work entitle us to expect an authoritative and standard textbook on leprosy. This expectation is to a considerable degree fulfilled in the present volume. The author covers the whole subject of leprosy from a practical standpoint. In the earlier chapters he classifies and describes both clinically and histologically the various phases of the complex leprotic process. The emphasis is laid particularly on the meaning and interpretation of these phases. The main part of the work is devoted to the treatment of leprosy and its varied complications. Here description and direction are clear, categorical and detailed. Readers will find a wealth of information on the diagnostic, surgical and medical procedures to be adopted in dealing with lepra reaction, ulceration and nerve involvement, and with leprosy of the eye, nose and throat. The literature on leprosy has tended to be curiously secretive about these subjects and Dr Cochrane's book fills a long-felt want. The latter part of the book deals adequately with the subjects of prevention and institutional care. There is a welcome emphasis throughout on the importance of child leprosy. The book is admirably illustrated.

This textbook is important enough to merit criticism. There are a number of printing errors. On page 124 the author promises an appendix on the preparation of ethyl esters—a promise which is not fulfilled. In the chapter on pathology there is too much exposition and also a tendency, not uncommon among pathologists as well as clinicians, to attribute motive and purpose to cell behaviour. For a textbook which merits wide acceptance there is occasionally too much personal emphasis and too great a stress on the work done in Madras. Readers would also have welcomed a more detailed study of the modern sulphone treatment.

As a practical aid to the understanding and treatment of leprosy this textbook will be found indispensable to the younger leprologist, as well as to not a few of the older. A review of the book would be incomplete without reference to the deep and warm humanity of the author in his approach to the human and social aspects of leprosy.

Miracle drug : the inner history of penicillin

By DAVID MASTERS. 1946. London: Eyre and Spottiswoode. Pp. 191; 21 figs. on 9 plates. 10s. 6d.

This is a readable volume written in the current style of popular journalism and illustrated with excellent photographs of the scientists chiefly concerned in the discovery and development of penicillin. The information is offered in a manner calculated to appeal to the lay public, which likes its scientists and their deeds to be dramatised in the heroic fashion. A great deal in the book is thoroughly familiar to pathologists and bacteriologists; but there is also a fair amount of little-known information. The account is accurate, surprisingly so in some details, and it pays a well-deserved tribute, not only to the work done in establishing the scientific truth about penicillin, but also to the pertinacity, resource and character displayed— and required—in having the drug produced in sufficient quantity to influence the medical history of the war. The book may be recommended for medical students and indeed for all who have still to learn that a triumph in research is not secured without phases of despair and drudgery as well as moments of inspiration.

Henrici's molds, yeasts and actinomycetes

By C. E. Skinner, C. W. Emmons and H. M. Tsuchiya. Second edition, 1947. New York: John Wiley & Sons; London: Chapman & Hall. Pp. xiv and 409; 136 text figs. $5.00.

Although bacteriology may be regarded as the offspring of mycology, these two closely related branches of microbiology have been allowed to develop as independent disciplines, to their mutual disadvantage. The late Dr Henrici, in 1930, published his *Molds, yeasts and actinomycetes*, designed to introduce mycology to the bacteriologist. The book achieved well-merited popularity, but the untimely death of the author and the onset of the recent world war delayed the preparation of a second edition. This task was eventually undertaken by the present editors, who are specially qualified to present the subject from both the mycological and the bacteriological standpoint. In view of the notable advances in mycology during the past decade, particularly in its medical aspect, including the production of antibiotics, it was found necessary to rewrite almost the entire book and to add two new chapters.

The simple style of the text and the lucid descriptions of the morphology, physiology, genetics and reproduction of the microfungi, leading up to discussions on classification, will appeal strongly to the reader to whom mycology is *terra nova*.

Technical procedures and the economic, industrial and medical *aspects* of mycology are adequately treated, while the descriptions of mycological methods used in diagnosis, and the discussions on allergy, serology and immunity in fungous infections will prove especially interesting to the medical bacteriologist. However, the book is not merely a work on medical mycology : it comprehends a much wider field, and the important sections on the chemical activities of the fungi, including the production of antibiotics, form one of its most valuable features.

Ample illustrations and bibliography and a full index complete a well-produced book.

Without stressing the danger of narrow specialism or the advantage of the broader outlook which places the speciality in true perspective, it may confidently be said that this book should find a place on the bookshelf of every bacteriologist.

Bacteria in relation to domestic science.

By C. E. Dukes. 1947. London: Geoffrey Cumberlege, Oxford University Press. Pp. viii and 240 ; 9 figs., frontispiece in colour.   12s. 6d.

This book is intended for students of domestic science and it could be read with equal profit by medical students. It presents its well selected facts simply, clearly and accurately, and maintains a true balance between the pathogenic and the beneficial activities of bacteria. Future editions could be improved by mention of *urinary typhoid carriers*, by noting that vomited material should be *preserved for investigation from cases of* salmonella food poisoning, by amendment of the statement (p. 190) that "duck eggs are always safe when boiled, fried or otherwise cooked", and by adopting a consistent style of bacterial nomenclature in place of the present mixture which combines correct with less desirable usages.

Microdiffusion analysis and volumetric error
By E. J. CONWAY. 1947. London: Crosby Lockwood & Son, Ltd.
Pp. xix and 357 ; 63 text figs. 21s.

Micro-diffusion analysis represents a real advance in modern analytical technique, especially in the realm of clinical and biochemical investigations. It seems strange that the underlying principle of allowing the gaseous product of a reaction to pass by diffusion, in a closed chamber, into a suitable absorbent or reactant should not have been used previously in analytical procedures. Absorption of gases into appropriate reactants by distillation and aeration methods has, of course, been the basis of many well-known and important chemical and biochemical methods of analysis, but the methods are usually tedious and extravagant in apparatus and material. Conway's technique involves the use of a simple and inexpensive glass apparatus (termed the " Unit ") consisting of an inner and an outer chamber. The gaseous product of a reaction (*e.g.* ammonia, volatile amines, carbon dioxide, acetaldehyde) passes from one chamber where the gas exerts a certain tension into an absorbent in the second chamber where the tension is zero. Analyses are made of the contents of the second chamber and the accuracy is limited only by the accuracy of delivering *and titrating fluid volumes of the order of 1 ml. Conway claims that* the method appears to be the " simplest possible consistent with the maximum attainable accuracy in the handling of micro-volumes ". The technique has been adopted for analyses of ammonia, amides and amines, adenosine and adenosine derivatives, halogens, alcohol, acetone, lactic acid and glucose, carbon monoxide and carbon dioxide. It may be also used for the assessment of the activities of enzymes, such as carbonic anhydrase, which control reactions giving rise to gaseous products.

Conway's book describes his " Unit ", the various physico-chemical factors involved in diffusion techniques, the micro-pipettes and burettes required and the errors involved in their use. It gives details of the analytical methods, involving the micro-diffusion technique, which have now been adopted in many laboratories, referring in particular to estimations of ammonia, urea, amines and lactic acid, etc., in biological fluids and tissues. Many of these methods seem to be particularly suitable for adoption in clinical investigations.

The book has a most valuable section on the errors involved in volumetric titrations. It has a good bibliography and is well illustrated and produced. It may be highly recommended to all engaged in biochemical and clinical laboratory practice.

PROCEEDINGS OF THE PATHOLOGICAL SOCIETY OF GREAT BRITAIN AND IRELAND

4th and 5th July 1947

The seventy-fourth meeting of the Society was held at the Royal Victoria Infirmary and the Department of Bacteriology, the Medical School, Newcastle-upon-Tyne, on Friday 4th and Saturday 5th July 1947.

Communications and demonstrations

The item marked with an asterisk is abstracted below

A. C. LENDRUM. Rhabdomyosarcoma of the diaphragm in a case of asbestosis.

H. ŠIKL. Addison's disease due to congenital hypoplasia of the adrenals in an infant aged 33 days.

J. B. DUGUID. Mural thrombosis as a factor in atherosclerosis.

J. G. THOMSON. Observations on Gram's stain.

W. BLACKWOOD. Brain-stem hæmorrhage secondary to acute intracranial hypertension.

DOROTHY S. RUSSELL and A. H. E. MARSHALL. Microgliomatosis : a form of reticulosis involving the central nervous system.

G. BUCKLE, P. MACCALLUM, H. A. SISSONS and J. TOLHURST. A new mycobacterial infection in man.

J. FRANCIS. Some observations on the behaviour of Lancefield's group-A and group-B streptococci in the chick embryo and mouse.

R. D. STUART. Further observations on the maintenance of the viability of the gonococcus during transport.

A. L. LATNER. Observations on a case of nocturnal hæmoglobinuria (Marchiafava-Micheli syndrome).

S. D. ELLIOTT. Properties and crystallisation of streptococcal proteinase.

*I. LOMINSKI and J. A. MILNE. Heat resistance and filterability of staphylocoagulase.

A. H. EMSLIE-SMITH. Agglutination of coliform bacilli by normal rabbit's serum : the complexity of the antibody concerned.

C. RAEBURN. Isolated myocarditis of diffuse type in an infant of eleven months.

FREDA K. HERBERT. Acute idiopathic porphyria.

W. P. WEIR. A case of epithelioma of the stomach.

E. S. HORNING. The induction of bronchiogenic carcinomas in mice.

I. RANNIE. Massive intravascular tumour growth without thrombosis in a case of uterine sarcoma.

I. RANNIE, H. G. H. RICHARDS and J. G. THOMSON. Isolated myocarditis.

J. G. THOMSON. Obstruction of main bronchus by plastic exudate in lobar pneumonia.

R. SCHADE. Peptic ulceration in a gastrogenous mediastinal cyst, with fatal hæmatemesis.

T. CRAWFORD. An unusual form of bronchial tumour.

J. S. FAULDS. Synovial sarcoma : three cases.

F. B. SMITH.   Probable apocrine carcinoma of breast.

C. J. E. WRIGHT.   Two cases of arachnoidal endothelioma (meningioma), one showing advanced myxoid degeneration, the other telangiectatic (" angiomatous ").

J. S. YOUNG.   A cinematograph film illustrating a single principle likely to be concerned in the dissemination of malignant tumours—by progressive and retrograde embolism and by the " permeation " of lymphatics and venules—and in the onset of paravascular necrosis.

Abstract

577 . 15 . 036 : 577 . 15 (staphylocoagulase)

HEAT RESISTANCE AND FILTERABILITY
OF STAPHYLOCOAGULASE

Iwo LOMINSKI and J. A. MILNE *

From the Department of Bacteriology, the University and Western Infirmary, Glasgow

In the course of work on anti-staphylococcal immunity there was need for large amounts of coagulase containing no living cells (filtered or heated cultures). The puzzling phenomenon was observed that coagulase of the same strain of *Staphylococcus aureus* gave from one experiment to another variable results as regards resistance to heat and filterability.   Similar conflicting statements are met with in the literature, *e.g.* von Gonzenbach and Uemura (1916), Gross (1931), Gengou (1933), Walston (1935), Fisher (1936), Lominski (1944) and Smith and Hale (1944).   The present work is an attempt to find the reason for these inconsistencies.

Five strains of *Staphylococcus aureus* were selected, all of which were killed when exposed to 65° C. for 45 minutes.   Cultures were made in horse-heart extract and horse-heart digest broth (*p*H 7·4), with and without the addition of human plasma.   Cultures were incubated for 1-14 days.   After incubation the *p*H of every flask was tested.   Seitz filters (Sterimat, grade S.B.) and Chamberland filters (L2, L2bis and L3) were used ; heating was effected by immersing the cultures in a water-bath at 65° C. for 45 minutes.   Citrated plasma plus 2 units of heparin per c.c. was used throughout.

Results

The *p*H of the cultures varies considerably with the type and batch of medium and, to a less extent, with the time of incubation.   Thus cultures in extract broth are almost always acid after 24 hours (down to *p*H 6·4) and usually remain so ; occasionally the *p*H rises and stays neutral after 5-7 days.   Certain batches of media first become acid (48 hours), then neutral and finally permanently acid.   Digest-broth cultures are alkaline (up to *p*H 7·8) after 24 hours and usually remain so on further incubation.   It was found that acid cultures yield a weak but definite coagulase on filtration (maximum titre 1 : 128), but none on heating, while alkaline cultures give on heating a strong coagulase

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(titre up to 1 : 8000) but none or only traces on filtration. In order to determine whether the reaction has a direct influence on filterability and heat resistance every culture after incubation was divided into four portions; the reaction of two was made acid (pH 6·0-6·8), of the other two alkaline (pH 7·0-7·8). One acid and one alkaline specimen were filtered, the other pair heated. Coagulase proved heat resistant at the alkaline and filterable at the acid reaction. It must be noted, however, that the titre of coagulase obtained by filtration through Seitz or Chamberland filters is always considerably lower than the titre of the heated portion. Thus in contrast with Gradocol membranes (Smith and Hale) Seitz and Chamberland filters retain the greater part of the coagulase. Also, even under optimum conditions, digest broth yields on heating more coagulase and on filtration less than extract broth. The results of an experiment are given in the table.

pH	Digest broth: titre		Extract broth: titre	
	after heating	after filtration	after heating	after filtration
7·6	1 : 8000	0	1 : 1000	0
7·0	1 : 4000	0	1 : 250	0
6·7	1 : 250	1 : 2	0	1 : 16
6·5	0	1 : 4	0	1 : 64
6·3	0	1 : 16	0	1 : 128

Contrary to these findings with whole cultures submitted to heating, the coagulase present in filtrates resists subsequent heating at both alkaline and acid reactions. The factor which causes heat sensitivity of coagulase in acid whole cultures is retained completely by Chamberland L3 and Seitz filters, but only incompletely by Chamberland L2 and L2bis candles. It was found that washed live staphylococci added to filtered coagulase were without effect on its heat resistance, whereas the addition of 10 per cent. of the supernatant fluid of a spun culture to filtered coagulase rendered it sensitive again to heating at acid reaction.

The addition of plasma to the medium increases considerably the filterability of coagulase, especially in extract broth, through both Seitz and Chamberland filters (cf. Gengou), titres of 1 : 4000 being frequent. Here again filterability is best at a pH below 7, heat resistance above 7. However, coagulase obtained by filtration of acid plasma-broth cultures remains heat sensitive. The increased filterability in plasma media appears to hold not only for coagulase but also for the unknown factor which determines the heat sensitivity of coagulase.

It may be concluded from the present experiments that, apart from affecting the actual production of coagulase, the medium has an influence on the filterability and heat resistance of coagulase. The pH determines, *ceteris paribus*, both filterability and heat resistance; heat resistance appears to be directly, filterability inversely correlated with the pH value. The conflicting results of previous work thus become explicable, since differences of media, filters and pH seem to have been overlooked, while whole cultures and filtrates have been employed indiscriminately for testing heat resistance.

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The Journal of
Pathology and Bacteriology

Description of the experiments

The details of the procedure were the same as in the last investigation (Callender *et al.*), with the same object of allowing both erythropoiesis and survival of the transfused cells to proceed under conditions as nearly normal as the method will allow. Blood was removed in two or three bleedings of the subjects and replaced by cells from an approximately equal volume of group-O blood. The transfused cells were then followed by the modified Ashby technique (Dacie and Mollison, 1943). The volunteers were four young women working in the department and two male medical students, none of whom had been transfused before. They all had Rh-positive blood. Apart from subject V they had *no reaction* to the transfusions and no irregular agglutinins could be demonstrated later in their sera.

Subject I. Female, group $A_1 Rh_1Rh_2$. Two healthy children. Menstruation 5 days in 25-30 days; *normal loss*. 1080 c.c. blood withdrawn. Transfused with cells from about 1100 c.c. group-O Rh+ blood. Hæmatocrit before experiment, 39; afterwards, 41.

Subject II.   Female, group A Rh₂rh.   Menstruation 4-5 days in 28 ; normal loss.   1310 c.c. blood withdrawn.   Transfused with cells from about 1350 c.c. group-O blood.   Two of the three donors Rh+, one Rh—.   Hæmatocrit before experiment, 39 ; afterwards, 42.

Subject III.   Female, group A₁ Rh₁Rh₂.   Menstruation 7 days in 28 ; loss heavy.   1170 c.c. blood withdrawn.   Transfused with cells from about 1200 c.c. of group-O Rh+ blood.   Hæmatocrit before experiment, 42 ; afterwards, 41. This subject fainted during the withdrawal of blood.
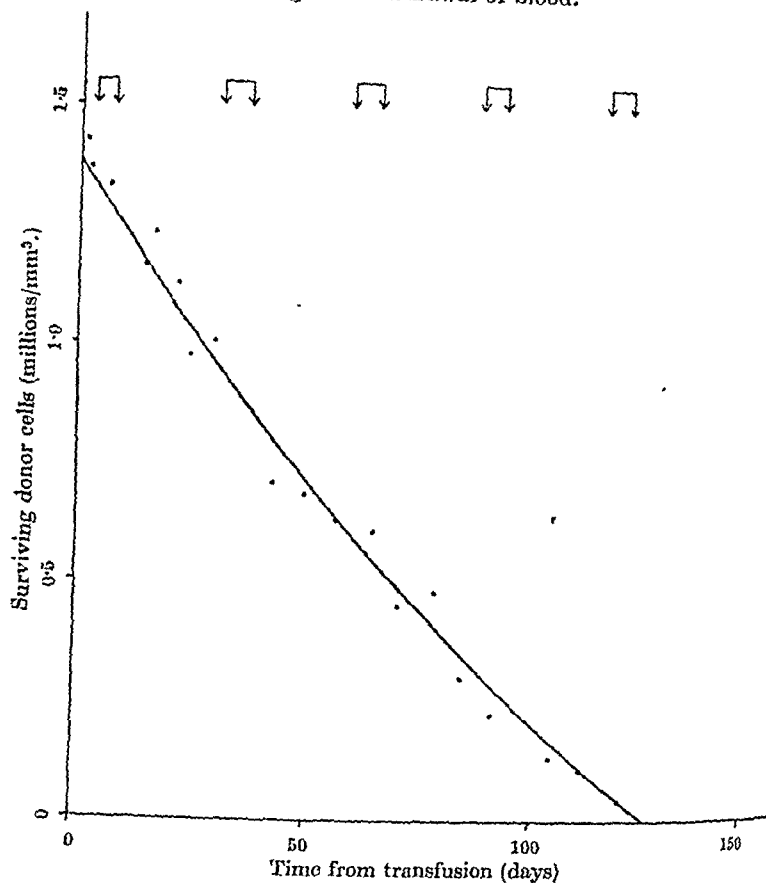
Fɪɢ. 1.—Survival of transfused cells in a normal female recipient (subject IV). The menstrual periods are indicated by yoked arrows.

Subject IV.   Female, group A₂ Rh₂rh.   Menstruation 5 days in 28 ; loss normal.   1210 c.c. blood withdrawn.   Subject felt faint.   Transfused with cells from about 1100 c.c. of group-O Rh+ blood.   Hæmatocrit before experiment, 42 ; afterwards, 39.

Subject V.   Male, group A₁ Rh₂Rh₂ or A₁ Rh₂rh.   1000 c.c. blood withdrawn. Subject felt faint.   Transfused with cells from about 1000 c.c. of group-O Rh+ blood.

Subject VI.   Male, group A Rh+.   1300 c.c. blood withdrawn.   Transfused with cells from about 1150 c.c. of group-O Rh+ blood.   This subject exhibited a post-transfusion reaction and the counts were subsequently very erratic. The result was therefore rejected as unreliable, although in fact it agreed with findings from more normal cases.   As with subject IV of our previous paper, this agreement is of some interest as showing the stability of the 120-day limit to the life span of the cell.

It may be said at once that the survival curves for women differed from those for men in an unmistakeable and regular manner. Typical examples are given in figs. 1 and 2. Consequently we have to refine our former quantitative treatment of the observations to take account at least of the known additional blood loss, namely menstruation, in women. Unfortunately in carrying out the experiments it was not possible to ensure that the blood transfused into any one subject was all derived from donors of one sex. The conditions of the experiment were thus not the simplest possible and the deductions we can make are thereby limited. In spite of the new features which have appeared, it is still true that the survival curves approach the axis fairly sharply at about
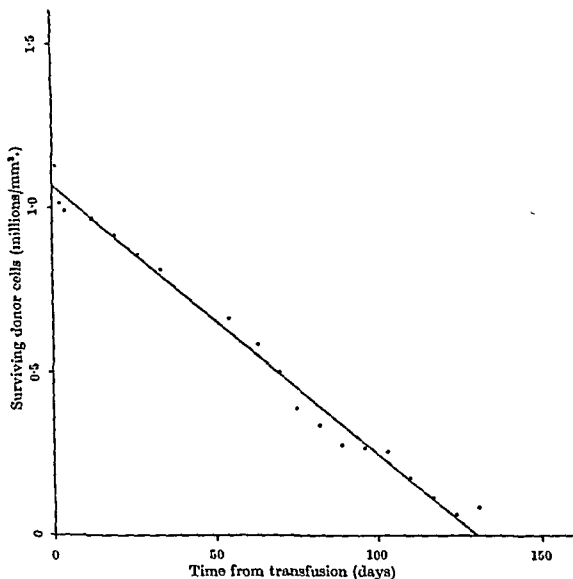
Fig. 2.—Survival of transfused cells in a normal male recipient (subject V).

120 days from transfusion. This confirms the general hypothesis that most cells persist for this length of time after leaving the bone-marrow and, more particularly, indicates that the biological machinery which brings it about is quantitatively similar in men and women. It may be suggested that the differences in the two classes of curves are due to external influences unconnected, or only remotely connected, with the internal economy of the red cell. Therefore, before considering the numerical results, we first obtain some mathematical relationships from the analysis of blood loss into the operation of two factors : (i) a factor which determines the destruction of the cell according to its age ; we shall refer to this as the " intrinsic factor " ; (ii) a factor which acts without regard to the cell's age and is possibly composed of several independent parts not kinetically distinguishable, e.g. mere physical escape of blood, pathological hæmolysis and casual trauma due to passage through the

capillaries. We shall assume this factor to be acting with constant intensity, the rate of removal of cells by it being proportional to the number available to its action. It will be referred to as the "extrinsic factor". It will be realised that the more detailed picture is subject to the same general uncertainties as are introduced by the assumptions we formerly discussed, apart from the special ones made here in developing it.

Cell survival under the influence of diverse destructive factors

In all cases we may write the fractional rate of loss of cells as the sum of the non-selective loss and the loss by ageing. Using ϕ with the previous meaning of expectation of survival, or fraction surviving, to age τ at least, we have in a normal circulation

$$-\frac{d\phi}{d\tau} = D\phi - \frac{\phi}{\phi_a} \cdot \frac{d\phi_a}{d\tau}, \qquad \cdot \quad \cdot \qquad \cdot \qquad I$$

where D is a constant measuring the intensity of the extrinsic factor, and ϕ_a is a function of τ giving the expectation of survival accorded by the intrinsic factor. Here $d\phi_a/d\tau$ is multiplied by ϕ/ϕ_a because the added loss due to the extrinsic factor leaves only this fraction of the cells available to the operation of the intrinsic factor. Integrating equation I with the conditions that $\phi(0)$ and $\phi_a(0) = 1$, we have

$$\phi = \phi_a(\tau) \exp(-D\tau) \qquad \cdot \qquad \cdot \qquad \cdot \qquad II$$

This ϕ, as well as being the expectation of life, is also (apart from a constant multiplier) the age distribution of the cells in the circulation at any time, provided they are being formed at a fixed rate, as we have constantly to assume. We note that if the cells are removed to a different environment, ϕ_a will by hypothesis be unaltered, but D may change.

Now if n is the rate of production of cells per unit volume of blood, there are present in this volume

$$n\phi_a(\tau) \exp(-D\tau)d\tau$$

cells of age between τ and $\tau+d\tau$. Let N_1 be the total red cell count, and suppose some of the blood be transfused, reaching an initial count N_0 in the recipient. Then the number of cells transfused from this age-group is

$$\frac{N_0}{N_1} n\phi_a(\tau) \exp(-D\tau)d\tau.$$

If N is the total number of cells surviving to time t after transfusion, we have with our previous notation $N = N_0\psi(t)$. Let us designate by $N_0\Delta_\tau\psi(t)$ the number of cells from the age-group under consideration which survive to time t. Immediately after transfusion

$$\Delta_\tau\psi(0) = n\phi_a(\tau) \exp(-D\tau)d\tau/N_1.$$

Later we shall have for the rate of loss from the group, by similar reasoning to that above,

$$-\frac{d}{dt}\left\{\Delta_\tau\psi(t)\right\} = R\Delta_\tau\psi(t) - \frac{\Delta_\tau\psi(t)}{\phi_a(\tau+t)}\cdot\frac{d}{dt}\left\{\phi_a(\tau+t)\right\} \qquad . \qquad . \qquad \text{III}$$

For the effect of the extrinsic factor in the recipient, here called R, will in general be different from that in the donor (D). The extrinsic factor acts on the cells without regard to their age, but ϕ_a continues as before, and the age of the cells is now $\tau+t$. Equation III gives on integration

$$\Delta_\tau\psi(t) = A \exp(-Rt)\phi_a(\tau+t)$$

where A is a function of τ. Putting $t = 0$ we find

$$A = n \exp(-D\tau)d\tau/N_1,$$

and so

$$\Delta_\tau\psi(t) = n \exp(-D\tau-Rt)\phi_a(\tau+t)d\tau/N_1.$$

We obtain the total number surviving by summing the groups of cells which were of all possible ages at the time of transfusion :

$$\psi(t) = \int_0^\infty \Delta_\tau\psi(t) = \frac{n}{N_1}\int_0^\infty \exp(-D\tau-Rt)\phi(\tau+t)d\tau$$

$$= \frac{n}{N_1}\exp\{(D-R)t\}\int_t^\infty \exp(-D\tau)\phi_a(\tau)d\tau \qquad . \qquad . \qquad \text{IV}$$

This is the general form of the survival curve which we measure. The velocity constant of the extrinsic factor may take two values, L_m (in men) and L_w (in women), and they may be expected to be unequal. R and D will each take either of these values—the same value when recipient and donor are of the same sex, different values when they are of opposite sexes.

If, as we have reason to believe, the intrinsic factor acts simply by causing the destruction of the cell when its age is about 120 days, we have, ideally, neglecting the scatter of individual life spans about this average,

$$\phi_a = 1, \ \tau_a > \tau > 0 \ ; \ \phi_a = 0, \ \tau > \tau_a$$

where $\tau_a \simeq 120$. Then equation IV becomes

$$\psi(t) = \frac{n}{N_1}\exp\{(D-R)t\}\int_t^{\tau_a}\exp(-D\tau)d\tau$$

$$= \frac{n}{N_1}\cdot\frac{\exp\{(D-R)t\}}{D}\left\{\exp(-Dt)-\exp(-D\tau_a)\right\}.$$

But N_1/n is the true average life of the donor's cells, given also by

$$\bar{\tau} = \int_0^\infty \phi d\tau = \int_0^{\tau_a} \exp\left(-D\tau\right) d\tau$$

$$= \{1 - \exp\left(-D\tau_a\right)\}/D, \qquad \qquad \text{V}$$

whence

$$N = N_o \psi(t)$$

$$= N_o \exp\left(-Rt\right) \frac{1 - \exp\left\{-D(\tau_a - t)\right\}}{1 - \exp\left(-D\tau_a\right)} \qquad . \quad \text{VI}$$

τ_a is of course the end-point of our survival curves and is the reciprocal of the number which we have called L_s, giving the rate of destruction by the intrinsic factor alone. Since we may expect L_m and L_w—the possible values of R and D—to be considerably less than L_s (and this is borne out in the results), we expand the exponential functions and neglect cubic and higher terms, when instead of equation VI we have to a sufficient degree of approximation :

$$N = N_0(1 - L_s t)\left\{1 - \frac{L_s(2R - D)}{2L_s - D} t\right\} \qquad . \quad \text{VII}$$

That is, the curves we obtain for the survival of transfused cells will in general be nearly parabolic, whatever the sexes of donor and recipient, and in fitting them to the data we have to determine three parameters, N_o, L_s and $L_s(2R - D)/(2L_s - D)$. The last we abbreviate to L_c, which may be regarded as a measure of the departure of the curves from linearity.

We have now to enquire how adequately the true survival laws are approximated by equations II and VI, remembering that blood loss in menstruation is not continuous but periodic. Taking the extreme case, we postulate a catastrophic loss of equal amounts of blood at equal intervals of time and determine the equation for ϕ corresponding to equation II, assuming as before that the intrinsic factor destroys no cell until it has reached its age limit τ_a or $1/L_s$. We write $L_w m$ for the proportion of the total amount of blood lost on each occasion, m for the time interval and ν for the greatest integral number of intervals in the life span as conditioned by the intrinsic factor. L_w is then the average rate of loss from the circulation which agrees with the meaning it had in the previous paragraph. Then the chance of a cell's surviving to a time just following a loss of blood is less than its prior value by $L_w m$ times that value, but otherwise the probability does not alter until at $\tau = 1/L_s$ the cell is destroyed. From this it is seen that the curve for ϕ is stepped, the heights of the successive steps above the axis being

$$1,\ 1 - L_w m,\ (1 - L_w m)^2,\ \cdots$$

Such a stepped curve is shown in fig. 3, the values of L_s, L_w and m being 0·0083, 0·003, and 30, respectively. Superimposed on it is the curve given by equation II with the same values of the parameters. The expectation of life of the cell in the present circumstances is not a fixed quantity, however, and it has two extremes, according as the cell has been delivered into the circulation just before or just after a loss of blood. These are given by the expressions

$$\text{lower limit}: \; m\sum_{0}^{p\,=\,\nu-1}(1-L_w m)^{p+1}+(1-\nu L_s m)(1-L_w m)^{\nu+1}/L_s$$

$$\text{upper limit}: \; m\sum_{0}^{p\,=\,\nu-1}(1-L_w m)^{p}+(1-\nu L_s m)(1-L_w m)^{\nu}/L_s$$

With the same numerical values as in fig. 3, these extremes are 95·3 days and 104·8 days, while equation II gives 100·7 days. The
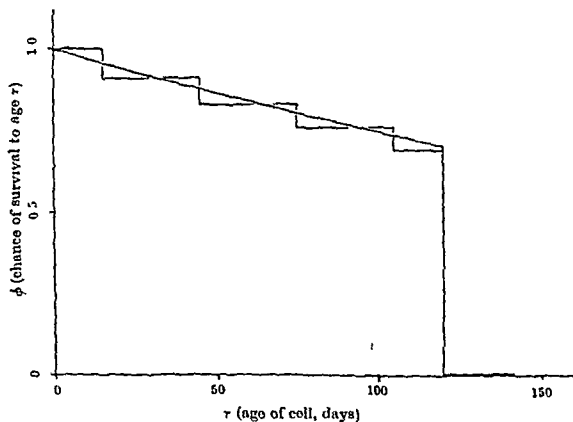
Fig. 3.—Comparison of continuous and discontinuous laws of survival.

differences are not large compared with the uncertainty of experimental results, in spite of the large value assumed for $L_w m$ (9 per cent.. or about 450 c.c. per period); also the loss in practice is not instantaneous but spread over some days. It is evident, then, that we may safely use the more convenient equation II as an approximation in numerical work. Exactly similar reasoning shows that equation VI or VII is adequate to represent the survival of transfused cells in a female subject.

30 per cent., 250 c.c. is still excessive. It is true that it probably errs systematically by excess : in a sensibly linear curve, a rapid initial fall of small duration and a slight tailing off at the end will be neglected, but they will be included in the overall curvature if the latter is everywhere appreciable. Thus the estimate of the difference between the two types of survival curve will be too high. Scatter of the life span of the cells about the modal value τ_a would produce a tailing off in the curve of a different character from the steady decrease in slope found here. Increase in degree of scatter will not account for the apparently greater rate of cell destruction in women. In another respect the result is not unreasonable. The total red-cell count is normally lower in women than in men. Vaughan (1936) gives the respective average values 5·01 and 5·42 millions/mm³. The lower count might, a priori, be due (i) to a greater rate of cell destruction; (ii) to a smaller influx of new cells resulting from a lower marrow activity or from a relatively smaller amount of erythropoietic tissue; (iii) to both these factors together. Our results should enable us to decide between these possibilities and they do in fact lead to an acceptable answer. The rate of production of cells per unit volume of blood is the ratio of the red-cell count to the average life, $n = N_1/\bar{\tau}$, and a comparison between men and women can be made thus :

$$\frac{n_w}{n_m} = \frac{N_{1w}}{\bar{\tau}_w} \times \frac{\bar{\tau}_m}{N_{1m}} \qquad . \qquad . \qquad . \qquad . \qquad \text{VIII}$$

We have found that blood destruction is greater in women than in men, and the ratio of the red-cell counts is known, but we cannot find L_w and L_m separately with the data we have. However, the ratio of the average lives can be found with sufficient accuracy for the present purpose ; from equation V,

$$\frac{\bar{\tau}_m}{\bar{\tau}_w} = \frac{\{1 - \exp(-L_m/L_s)\}L_w}{\{1 - \exp(-L_w/L_s)\}L_m} \simeq 1·17.$$

Substituting in equation VIII,

$$\frac{n_w}{n_m} = \frac{5·01}{5·42} 1·17 = 1·08.$$

The difference of this ratio from unity is not really significant. To a first approximation we may say that the lower red-cell count in women is largely due to a greater rate of cell destruction and that the rate of blood formation is nearly the same as in men. Our estimates are of course all made relative to unit volume of blood and do not represent absolute total amounts.

An attempt was made to detect a fluctuation in the survival curves for the female subjects by correlating the apparent errors of the data with the menstrual periods. It was found that the former were erratic and on the whole larger than the greatest possible

irregularity that could be due to the latter, and no significant period could be found. Fig. 4 (subject II) is given as an example. The stepped line bears the same relation to the fitted parabola as does the stepped to the smooth curve in fig. 3 : instead of a steady loss of cells at a rate NL, per day to the extrinsic factor, it represents the loss between two menstrual periods as occurring suddenly at the onset of the first of the two, with only the normal-ageing proceeding
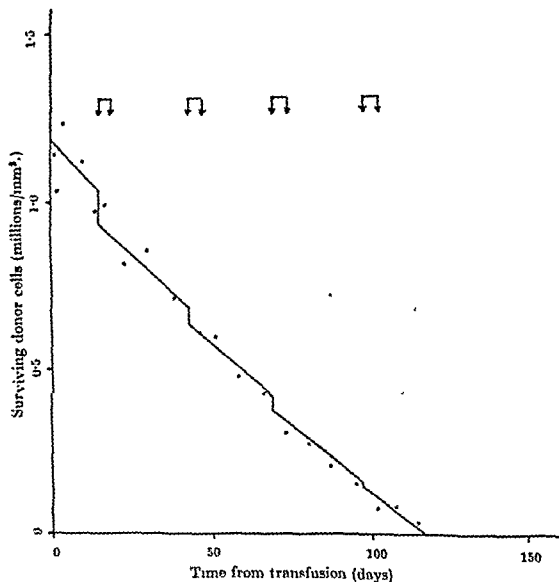
FIG. 4.—Survival of transfused cells in a normal female recipient (subject II), with a discontinuous law of survival for comparison. The menstrual periods are indicated by yoked arrows.

in the intervals. The result stated above is immediately obvious and, a fortiori, we could not expect the true periodic blood loss to be detectable.

Discussion

In this paper we have been largely concerned with a more detailed, and therefore more exceptionable, account of the survival of red cells. It is all the more important that the broad outline given by our former treatment should be sound. It has in fact been confirmed in two recent investigations by different methods.

and appreciably curved in women.   Their average lives were respectively 63 and 54 days.

3. The relation has been deduced between the decay of transfused cells and the law of survival of the individual erythrocyte on the assumption that two types of destructive factor are normally operative.

4. It is concluded that most red cells live for approximately the same time, 120 days, in both men and women.

5. Extraneous factors cause the loss of some cells before they have reached this age-limit. The loss is greater in women than in men by the equivalent of 400 c.c. of blood per month—more than can be ascribed to menstruation.

6. The average age of cells at death is tentatively assessed at 90-100 days in women and 110-120 days in men.

REFERENCES

BAAR, H. S., AND LLOYD, T. W. .   1943. Arch. Dis. Childh., xviii, 1.
BARER, A. P., AND FOWLER, W. M.   1936. Amer. J. Obst. Gyn., xxxi, 979.
CALLENDER, SHEILA T., POWELL,   1945. This Journal, lvii, 129.
  E. O., AND WITTS, L. J.
DACIE, J. V., AND MOLLISON, P. L. 1943. Lancet, i, 550.
JEFFREYS, H. . . . . . .   1939. The theory of probability, Oxford. chaps. V and VI and appendix I.
JOPE, E. M. . . . . . .   1946. Brit. J. Industr. Med., iii, 136.
MILLS, J. N. . . . . . .   1946-47. J. Physiol., cv, 16P.
SHEMIN, D., AND RITTENBERG, D.   1945. J. Biol. Chem., clix, 567.
VAUGHAN, JANET M. . . . .   1936. The anæmias, 2nd ed., London, pp. 1 and 3.
WIDDOWSON, E. M., AND McCANCE,   1942. Lancet, i, 588.
  R. A.

Addendum

Since this paper was submitted for publication further work has appeared which corroborates our results. Shemin and Rittenberg (1946) have now completed an experiment with labelled glycine on the lines of that described above (p. 530). They find the average value of the life span to be 127 days. Also experiments with radioactive iron and phosphorus indicators (von Hevesy. 1947) point to the same conclusion.

REFERENCES

VON HEVESY, G. . . .   1947. Cited in the report of the Eleventh International Congress of Pure and Applied Chemistry, Nature, clx, 246.
SHEMIN, D., AND RITTENBERG, D.   1946. J. Biol. Chem., clxvi, 627.

A NEW SALMONELLA (*SALM. FAYED*) WHICH CAUSED FATAL ENDOCARDITIS IN MAN

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FATAL disease from lesions other than those of the gastro intestinal tract is not commonly caused by members of the Salmonella group apart from *S. typhi* and the paratyphoid bacilli.

CASE REPORT

The patient, a German prisoner of war, complained of acute diarrhœa on 31.8 45. He was admitted to the sick bay and three days later to the P.O.W. wing of no. 19 General Hospital, stationed in Egypt. On admission his general condition was fair, temperature 100° F., pulse 84. Nothing abnormal was noted in the chest other than increased rapidity of the heart. On examination it was found that there was tenderness on pressure in the left lower quadrant of the abdomen There was no enlargement of the liver, the spleen was not palpable and there was no rash. The patient passed 15 fluid motions in 24 hours. He was treated with sulphaguanidine, 6 g. daily for four days. The diarrhœa gradually diminished until at the end of treatment he was passing one normal stool daily. However the temperature remained raised, though nothing abnormal was found on abdominal examination On the 18th day of illness the patient complained of pain in the right shoulder and right knee, but on examination no abnormal signs were found. A low pre systolic mitral murmur was heard on auscultation. The temperature was 99° F., pulse 104. A 3 day course of sulphaguanidine, 6 g. daily, was given but the temperature was unaffected

On the 30th day of illness the patient was examined by the British medical specialist, who reported that he looked pale and ill and had clubbing of the fingers No petechiæ were seen The spleen was enlarged to two fingers-breadth below the costal margin. The pulse was not of the Corrigan type A pronounced aortic systolic murmur and possibly a pre systolic murmur at the apex were heard A provisional diagnosis of bacterial endocarditis was made.

During the sixth week of illness the patient suddenly complained of pain in the left foot, which was found to be pulseless, and colder to touch than the right The heart sounds were unchanged Sulphadiazine was given but the patient's condition was unaffected. During the ninth week the patient suddenly complained of severe pain in the left upper quadrant of the abdomen and a diagnosis of splenic infarct was made. The patient's condition now gradually deteriorated, the heart becoming progressively enlarged until he died, 12 weeks after the onset of symptoms.

LABORATORY INVESTIGATIONS

From a blood culture taken during the 4th week of illness an organism of the Salmonella group was isolated. Blood cultures taken during the 2nd, 5th and 9th weeks were sterile. The urine was examined microscopically on many occasions with the following results: albumin present, erythrocytes ++. leucocytes +++, granular casts +++.

Blood examination showed a mild anæmia with a total white-cell count of 6000 per c.mm.: the differential count gave a normal distribution of leucocytes.

Post-mortem findings

The body was wasted and pale, the left foot bluish, with scaling hypertrophic skin. A specimen of blood for bacteriological investigation was taken by cardiac puncture.

Lungs. Basal congestion and purulent bronchitis.

Heart. The pericardium was densely adherent over the front of the heart. The lateral and posterior portions of the pericardium were free and there was a normal quantity of pericardial fluid. The heart was greatly enlarged, the left ventricle being dilated and hypertrophied. The right ventricle was also dilated, but only slightly hypertrophied. The aortic valve was congenitally deformed, showing two complete cusps and one which was less than 1 cm. in width. Large firm vegetations up to 1½ cm. in length were attached to the margins of all the cusps and the anterior cusp was ruptured. The coronary arteries were normal. No other noteworthy valvular abnormalities were found.

The femoral artery contained a partly organised embolus at the level of the profunda femoris.

Spleen. This organ was considerably enlarged and adherent to the parietal peritoneum and omentum at its lower pole. Underlying the adhesion was an abscess 3 inches in diameter, full of reddish-brown, foul-smelling pus. A specimen of the pus was taken for culture.

Suprarenals. Normal.

Kidneys. The left kidney showed a healed infarct: the right presented evidence of recent infarction over an area 1 inch in diameter in the cortex near the upper pole.

Histology

Aortic valve. The entire ventricular face of the valve was covered by structureless thrombus in which masses of Gram-negative bacilli were embedded. The free surface of the thrombus was irregular and a thin layer of leucocytes was adherent to it: the attached surface merged into the substance of the valve cusp, and there was some fibrous organisation of the deep aspect of the vegetations. Although

involved to a much less extent, the aortic aspect of the cusp also showed the presence of vegetations.

A projection of the vegetation extended for a considerable distance in the direction of the blood flow, forming a spur reaching into the first part of the aorta. There was marked distortion of this cusp and a considerable amount of round-cell infiltration and vascularisation at its base.

Kidney. The section showed a large infarct in which the renal tissue had preserved its general structure, but the cell outlines and nuclei had disappeared, leaving an area composed largely of necrotic eosinophil material. A heavy infiltration with polymorphs was present at the periphery of the necrotic zone and many extravasated red cells were seen between the tubular remnants. At the side of the large infarct two small arteries were found whose lumina were completely occluded by fibrin plugs containing large numbers of leucocytes and red cells. Each contained a single mass about 50 μ in diameter composed of Gram-negative bacilli.

Bacteriology

Blood culture was performed on four occasions during life, and at post-mortem, but only one, that of 5th October 1945, yielded a Gram-negative coliform bacillus. A similar organism was grown from the splenic abscess found *post mortem*. Subsequent investigation showed that these two bacteria were morphologically, culturally and serologically identical.

The organism was a motile Gram-negative rod. On plain and MacConkey's agar the colonies were 1–2 mm. in diameter, with a smooth surface and an entire edge. The biochemical reactions were as follows. Acid and gas were formed in glucose, mannitol, maltose, dulcitol, xylose, sorbitol, rhamnose, dextrin, trehalose and arabinose. Lactose, sucrose, salicin, adonitol and inositol were not fermented. Indole was not formed, the Voges-Proskauer reaction was negative, and the methyl-red and citrate-utilisation tests were positive. Slight acid was produced in litmus milk. Gelatin was not liquefied.

Serology

The organism was agglutinated by *S. newport* O antiserum to full titre and a *S. newport* O suspension was agglutinated to full titre by an antiserum prepared against the organism. It was further demonstrated that absorption of *S. newport* O antiserum by the new organism removed all O agglutinins to the homologous type and that *S. newport* was capable of removing all O agglutinins from an antiserum prepared against the new strain. This showed that the somatic make-up was VI, VIII.

The organism was in phase 2 on primary isolation, but it was soon

apparent that it was naturally diphasic, and that a broth culture normally contained both phases. It was agglutinated by *S. london* phase 1 (lv) antiserum, but whereas the homologous titre was 1 : 8000, agglutination of the new strain occurred only to 1 : 4000, suggesting that the two phase 1 complexes were not identical. But a serum prepared against phase 1 of *S. dar es salaam* (lw) gave agglutination to full titre with the strain under investigation. Similarly, an antiserum to the unknown strain agglutinated *S. dar es salaam* to full titre and it was shown that reciprocal absorption of agglutinins from the heterologous antisera by *S. dar es salaam* phase 1 and the new type in phase 1 was almost complete. This established that the major phase 1 components were lw.

Phase 2 was proved by pure single-component sera to contain factors 1, 2 and 3. *S. newport* var. *puerto-rico* is permanently in phase 2, whose constituents are 1, 2 and 3, and the new organism was agglutinated to full titre by an H antiserum prepared against *S. newport* var. *puerto-rico*. Conversely the antiserum against the new type gave agglutination to full titre with an H suspension of *S. newport* var. *puerto-rico*. Further examination showed that each organism was able to remove entirely the phase 2 agglutinins from the heterologous antiserum. Thus the phase 2 formula was 1, 2, 3.

The complete antigenic formula of this type is therefore VI, VIII . . . lw . . . $\longleftarrow\longrightarrow$ 1, 2, 3 . . . It was isolated at Fayed near the Great Bitter Lake in Egypt and we suggest the name *S. fayed*.

DISCUSSION

Generalised infection with organisms of the Salmonella group (excluding the recognised typhoid and paratyphoid types) is rare in comparison with the incidence of uncomplicated food poisoning. The commonest organism of this group to give rise to blood invasion is *S. cholerae-suis*. Harvey (1937), in a survey of cases reported up to that date, found that only 3·3 per cent. of cases infected with this Salmonella suffered from a general infection. The disease has a considerable case-fatality rate, and Harvey's figures range from 19 per cent. in his own series to 39 per cent. in a series collected from other sources. An illness of the typhoid type occurred in 62 per cent. of all the cases he studied, and the remainder showed localisation in various sites such as lung, bone and joint. He cited one probable case of bacterial endocarditis caused by this organism; this was originally described by Gouley and Israel (1934), but as the patient recovered, final proof of infection of the cardiac valves was lacking.

Fatal bacterial endocarditis due to *S. cholerae-suis* was reported by Forster (1939), Read (1939) and Goulder *et al.* (1942). None of these patients gave a history of gastro-enteritis and the portal of entry was not apparent. Most of them showed evidence of pre-existing damage to the affected cardiac valves. In the two cases

reported by Forster, one patient showed syphilitic changes in the aorta and myocardium; the other presented evidence of previous rheumatic disease. The case reported by Read had no apparent previous valvular abnormality, but that of Goulder *et al.* showed rheumatic changes.

Septicæmic infections with *S. enteritidis* have been reported. They are not infrequently associated with meningitis, especially in infants (Guthrie and Montgomery, 1939), and occasionally with cholecystitis. Bacterial endocarditis caused by this type does not appear to have been recorded.

S typhi is a rare cause of bacterial endocarditis, and Meyer and Howell (1938) demonstrated that *S. paratyphi* B was capable of giving rise to a similar condition; evidence of previous rheumatic endocarditis was found in their case.

The case described by us presents certain interesting features. A definite history of enteritis was obtained, establishing the probable portal of entry. Blood invasion resulting in infection of the aortic valve occurred at an early stage in the disease, since the onset of the enteritis was on 31st August 1945, and the diagnosis of bacterial endocarditis was made on 29th September. During this period the patient was constantly febrile. The common association of congenital deformity of the aortic valve and its subsequent infection was apparent in this case.

Finally, the causal organism was a new type of Salmonella, which was first isolated from the blood after the initial enteritis had subsided. It is noteworthy that *S. fayed* was grown in pure culture from both the blood and the splenic abscess, thus indicating that it was the sole infective agent concerned.

SUMMARY

A description is given of a fatal case of bacterial endocarditis caused by a new type of Salmonella, *S. fayed*. This organism has the antigenic formula VI, VIII . . . Iw . . .◄——►1, 2, 3 . . .

REFERENCES

FORSTER, D. L. . . . . . . 1939. *Amer. J. Med. Sci.*, excvii, 234.
GOULDER, N. E., KINGSLAND, MARGARET F., AND JANEWAY, C. A. 1942. *New Engl. J. Med.*, ccxxvi, 127.
GOULEY, B. A., AND ISRAEL, S. L. 1934. *Arch. Int. Med*, liii, 699.
GUTHRIE, KATHARINE J., AND MONTGOMERY, G. L. 1939. *This Journal*, xlix, 393.
HARVEY, A. M. . . . . . . 1937. *Arch. Int. Med.*, lix, 118.
MEYER, J., AND HOWELL, KATHARINE M. 1938. *Arch. Path.*, xxvi, 368.
READ, C. T. . . . . 1939. *J. Inf. Dis*, lxv, 263.

LIVER ATROPHY PRODUCED BY CHRONIC SELENIUM INTOXICATION

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(PLATE LXXIII)

THE recent description by Earle, Small and Victor (1942) of liver atrophy uncomplicated by necrosis or fibrosis in rats fed with excessive amounts of dl methionine encourages me to describe some experiments carried out in 1940 which bear on this interesting topic. Whilst attempting to produce liver cirrhosis with selenium salts, I encountered a pure form of liver atrophy, confined to the right lobes and associated with hypertrophy of the left lobes, the organ assuming a distorted and sometimes bizarre shape.

METHODS

Eighteen male albino rats, average weight 185 g., were given twice weekly subcutaneous doses of sodium selenite as a 0.1 per cent aqueous solution for a period of 140 days. The initial dosage was 1.6 mg./kg. body weight. This was increased from time to time until towards the end of the experiment the twelve survivors were tolerating 12 mg./kg. Since 3.5 mg./kg. is the LD_{50} for single administrations, it is obvious that a tolerance had been acquired and the animals were existing on doses equivalent to more than three times a fatal dose. It cannot be said that such tolerance was complete for all had descended to a level of body weight much lower than their initial healthy level and presented signs of chronic intoxication. Throughout the experimental period, the rats were maintained on a laboratory diet of Rowett Institute cubes (Thomson, 1936) given in unlimited amount, and of which they ate voraciously. A control series of 10 rats receiving the same kind of food grew steadily throughout the same period. All rats were kept in wire cages in groups of 5 or less. Strict asepsis was maintained with the injections and in one animal only was there sepsis in the injected areas.

Injections were stopped at the end of 140 days and 6 animals were allowed to survive for a further 60 days.

Body weights were recorded at regular intervals and the means for the 12 survivors are shown in fig. 1. There was a remarkably steady behaviour in all instances. A careful autopsy was made on each animal, followed by a microscopical examination of paraffin embedded material stained with Ehrlich's acid hæmatoxylin and eosin, Weigert's iron hæmatoxylin and van Gieson, Mallory's aniline blue method for connective tissue and Gomōri's reticulum method, together with fat staining of frozen sections, from heart, lungs, liver,

of the poison employed at that period. With cessation of injections the remaining animals steadily gained in weight. A striking feature during the period of weight loss was the enormous appetite of the animals. Clearly, loss of weight cannot be attributed to starvation in the sense of failure to eat. Some metabolic disturbance seems to be indicated, but I have no idea what this may be. Loss of fluid with dehydration cannot be the main factor, for diarrhœa was present in two animals only and then only towards the end of the course. Water was available to all the rats but none exhibited abnormal thirst during the daytime.

Skin disturbances, with dryness, scaliness and falling out of hair, were prominent, large patches of skin becoming bald as the intoxication developed. Some yellowish-brown pigmentation of such areas was noticeable. Bone lesions were not apparent.

The chief pathological findings were localised liver atrophy, atrophy of the spleen and to a less extent of the sex organs and thymus, occasionally of the small intestines. The other organs were normal. Ascites was not found but pleural effusion occurred in fatal cases. The liver changes were by far the most striking, the organ being distorted, often with large hypertrophied left lobes, shrunken right lobes, a smooth pale surface and rounded edges. Some examples of this are shown in fig. 2. In two animals dying after 101 and 118 days, the liver looked like a huge heart through downward hypertrophy of the left middle lobe and almost complete atrophy of the other lobes. The capsule in some was thickened and in 3 cases vascularised adhesions between the inferior margin and an adjacent loop of gut existed. There was no macroscopical evidence of liver necrosis or fibrosis and the bile passages were normal. The organ was firm, though pliable and cut easily. Microscopical examination of the liver failed to disclose any pathological change except atrophy of liver cells in the affected lobes, together with hypertrophy of the cells in the other lobes. Measurement of 1000 liver cells from microscopical fields selected at random from atrophic areas disclosed considerable shrinkage as compared with cells in normal control livers. Nuclei were also smaller, often with variation in their staining: sometimes they were pyknotic. No fat was present in the liver cells except in one rat which died on the 118th day. Bile ducts and blood vessels were normal. The thickened capsules showed collagen fibrils with very few cells. The portal canals were closer set than normal. One animal presented a little condensation fibrosis spreading outwards from some of the portal canals. Atrophying lobes tended to fuse, their boundaries being indicated by the fibrous capsules only. The hypertrophied lobes contained numerous large cells with large nuclei scattered throughout the lobules in no recognisable pattern, but mitotic figures were not numerous.

The spleen showed atrophy of lymphoid tissue, with small Malpighian bodies, some thickening of the capsule, a little increase

in pulp fibrils and often much iron-free pigment. There was no necrosis and no vascular lesion or hæmorrhage. The portal and splenic veins were carefully dissected but presented no abnormality. The infrequently occurring changes in the sex glands and intestines appeared to be those of simple atrophy.

DISCUSSION

Repeated subcutaneous administration of increasing amounts of sodium selenite to rats has been accompanied by cessation of growth and loss of weight, skin changes, striking atrophy of the right lobes of the liver with hypertrophy of the left lobes, a slight to moderate degree of atrophy of the spleen and inconstant atrophy of the sex glands. Food intake was well maintained despite the intoxication, and fluid loss by the excretory routes was not excessive. In some cases death occurred. Discontinuance of the toxic agent led to recovery.

The liver changes are interesting, for they suggest a true atrophy localised to particular regions of the organ and are presumably the result of intoxication with a selenium salt. Atrophies resulting from chemical action must be rare and so far as I know are not listed amongst the usually accepted causes of this condition. It is worth while considering whether the liver atrophy is due to the poison. The most obvious suggestion is that it is a nutritional effect, for wasting of the liver sets in fairly quickly and may become pronounced when there is starvation and malnutrition (Jackson, 1925, 1929 a and b). My rats were maintained on a diet which control experiments showed to be adequate for growth and health. Moreover, the experimental rats displayed no loss of appetite : on the contrary, they ate ravenously and disposed of large amounts of food. At autopsy their stomachs and intestines contained much food residue. It is possible, of course, that absorption may have been interfered with or metabolism impaired. The liver atrophy, however, is of a type different from that usually described with inanition, for its localised character contrasts with the generalised wasting which apparently develops in starvation. Rather it suggests the action of a poison, either selectively on the liver cells or localised because of territorial differences in vascular distribution in the liver. Glynn and Himsworth (1944) have recently discussed the latter question and have brought forward evidence in its support. My impression is that similar factors may be concerned in these experiments with selenium. The enlargement of the left lobes of the liver is most readily explained as a compensatory hypertrophy. In the early stages of intoxication atrophy appeared to be more pronounced than hypertrophy. However, in one rat all the lobes of the liver were hypertrophied after 140 days' treatment, the liver weighing 10·8 g. as against a mean liver weight of 7·0 g. in normal rats of the same body weight. The absence of necrosis and fibrosis,

RAT LIVERS IN CHRONIC SELENIUM INTOXICATION

a

b

c

d

e
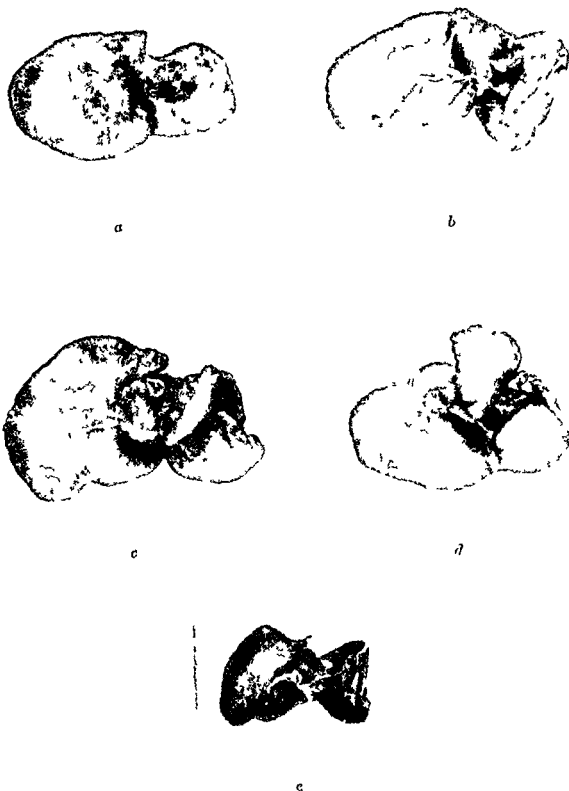
Fig 2—Atrophic livers from five of the surviving rats killed after 140 days showing shrunken right and large hypertrophied left lobes None of these livers show fibrosis microscopically Natural size

associated with the fact that acute sodium selenite poisoning does not lead to liver necrosis, inclines me to the view that there has been a steady, localised wastage of liver cells rather than any massive destruction and that this in time has brought about gross atrophy. Whether such a wastage is the result of direct injury of liver cells whereby they no longer grow or are not replaced when they die off, or whether it is the sequel of some obscure metabolic disturbance cannot be answered with the available information, but I am inclined to support the first view.

It is now known that the selenium of chronic poisoning exists in combination with the tissue proteins, especially the globulins of the plasma and liver (Smith, Westfall and Stohlman, 1938 ; Westfall and Smith, 1940), as well as non-protein compounds containing sulphydryl groups. Some selenium compounds, including sodium selenite, destroy certain oxidation catalysts (Bernheim and Klein, 1941), especially succinoxidase, the dehydrogenase part of the enzyme which contains —SH groups being inactivated. Selenite also inhibits the respiration of tissue slices of brain and liver (Wright, 1939, 1940). An acceleration of oxygen uptake precedes inhibition in the liver and aerobic glycolysis increases. Possibly through these disturbances liver-cell growth is interfered with and atrophy follows. On the other hand, various investigators have described lesions of the alimentary canal (see review by Moxon and Rhian, 1943) which disturb food absorption. Such changes were not impressive in my rats and were late in developing. The moderate degree of atrophy of the spleen might very well be the result of the liver changes.

This selenium atrophy differs from the form of atrophy attributed to excess of dl-methionine (Earle *et al.*), which may develop after a few days' feeding with methionine, and seems to be a general shrinkage of the liver from extreme atrophy of widely distributed liver cells. As with selenium atrophy, there is neither necrosis nor degeneration, fibrosis, bile duct proliferation nor jaundice. The spleen shows fewer Malpighian bodies and fewer pulp cells, with condensation of pulp structures. Sometimes there is atrophy of the kidneys. Loss of weight, too, is severe. Earle *et al.* suggest that the liver atrophy may be a reaction to increased metabolism consequent on methionine feeding, which they have demonstrated in the excised liver of dl-methionine rats. Of course, some similar explanation might apply in the case of sodium selenite, but it is idle to speculate at the present juncture.

In recent years much investigation of the toxicity of selenium and its compounds has been carried out in connection with " alkali disease " in cattle and sheep. This appears to be due to traces of selenium in indigenous grains and forage and is characterised by erosions of the weight-bearing bones, abnormalities of the hooves, stunting of growth, loss of body hair, impairment of reproductive power and severe anæmia. Pathological changes include necrosis and regeneration of

hepatic tissue, with extensive periportal fibrosis and cirrhosis, combined with hæmorrhages and secondary inflammation in the kidneys (Stenn, 1936 ; Painter, 1941 ; with reviews of the literature: Moxon and Rhian, 1943). Franke (1934), Franke and Potter (1934), Lillie and Smith (1940) and Smith *et al.* (1937, 1940) produced extensive liver necrosis and eventually nodular regeneration and cirrhosis by feeding rats with selenium salts, especially when the diet was poor in protein. Ascites and pleural effusions were common, jaundice developed, and degeneration in the thymus and sex organs was seen. This liver picture is in contrast to the one I have obtained by administering sodium selenite subcutaneously. Nevertheless it would appear that in both cases there was extensive liver-cell injury, the difference being one of extent and progress of damage. In my animals the process was spread over a relatively long period, a few cells at a time being destroyed Eventually the cumulative effect of this led to considerable atrophy, the animals dying in some instances, while in others compensatory hypertrophy of unaffected liver regions preserved the life of the animal. So far as I can tell from the descriptions, in the feeding experiments much larger tracts of liver were suddenly destroyed and a different pathological entity arose. It is possible that the difference might be the result of variations in the amount of selenium acting on the liver cells. With feeding experiments, presumably, absorbed selenium salts are carried directly to the liver by way of the portal circulation ; with subcutaneous injections considerable dilution and loss by adsorption may occur during their circulation through the lung capillaries, while only a portion of the selenium-containing blood thereafter passes directly to the liver by way of the hepatic artery, a great deal having to circulate through another set of capillaries before returning to the liver through the portal vein. Whatever the explanation of the divergence, an interesting contrast is afforded in the production on the one hand of pure liver atrophy and on the other of severe liver necrosis passing on to cirrhosis following prolonged administration of the same poison by different routes.

SUMMARY

Simple atrophy and hypertrophy of the liver without fibrosis is recorded in rats receiving steadily increasing subcutaneous doses of sodium selenite. Tolerance to selenium resulted in the course of the experiments. Reasons for this curious behaviour are discussed.

REFERENCES

Bernheim, F., and Klein, J. R.	1941.	J. Biol. Chem., cxxxix, 827.
Earle, D. P., Jr., Smull, Katharine, and Victor, J.	1942.	J. Exp. Med., lxxvi, 317.
Franke, K. W.	1934.	J. Nutrition, viii, 597.
Franke, K. W., and Potter, V.	1934.	Ibid., viii, 615.
Glynn, L. E., and Himsworth, H. P.	1944.	This Journal, lvi, 297.
Jackson, C. M.	1925.	The effects of inanition and malnutrition upon growth and structure, Philadelphia.
„	1929a.	Arch. Path., viii, 1042.
„	1929b.	Ibid., viii, 81 and 273.
Lillie, R. D., and Smith, M. I.	1940.	Amer. J. Path., xvi, 223.
Moxon, A. L., and Rhian, M.	1943.	Physiol. Rev., xxiii, 305.
Painter, E. P.	1941.	Chem. Rev., xxviii, 179.
Smith, M. I., Westfall, B. B., and Stohlman, E. F.	1937.	Publ. Hlth. Rep., Washington, lii, 1171.
„ „ „ „	1938.	Ibid., liii, 1199.
„ „ „ „	1940.	Nat. Inst. Hlth. Bull., clxxiv, 21.
Smith, M. I., Stohlman, E. F., and Lillie, R. D.	1937.	J. Pharmacol. Exp. Therap., lx, 449.
Stenn, F.	1936.	Arch. Path., xxii, 398.
Thomson, W.	1936.	J. Hyg., Camb., xxxvi, 24.
Westfall, B. B., and Smith, M. I.	1940.	Publ. Hlth. Rep., Washington, lv, 1575.
Wright, C. I.	1939.	J. Pharmacol. Exp. Therap., lxvi, 43.
„	1940.	Ibid., lxviii, 220.

EXPERIMENTAL SERUM CARDITIS AND ITS RELATIONSHIP TO RHEUMATIC FEVER

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(Plates LXXIV-LXXVII)

THE continued failure to isolate any organism which is acceptable as the cause of acute rheumatic fever has led to the development of various hypotheses as to the pathogenesis of this disease. Amongst these, much stress has been laid upon a possible allergic mechanism. Menzer (1902) was one of the earliest workers to suggest that rheumatic fever was the result of reaction in a predisposed person to various microbes, more especially streptococci. Vaubel (1932), Klinge (1933) and Junghans (1933-34) sensitised rabbits with small doses of horse serum by various routes and claimed they had thereby produced a cardiac lesion in every way similar to the specific lesion of rheumatic fever. Aschoff (1935), however, denied that any experimental lesion so far produced had succeeded in duplicating the morphological structure of the rheumatic nodule. In spite of this pronouncement the allergic theory has not been discarded.

It has been noted that serum sickness often produces signs and symptoms resembling those of acute rheumatic fever, and in two fatal cases Clark and Kaplan (1937) reported cellular infiltrations in the heart composed of mononuclear histiocytes and showing a similar distribution to those seen in rheumatic carditis. The occasional occurrence of polyarteritis nodosa as a complication of rheumatic fever has been reported by Friedberg and Gross (1934) and others. Recent observations by Rich and Gregory (1943) and by Rich (1945) have demonstrated that polyarteritis can be the result of sensitisation. Hence this association of diffuse arterial lesions with rheumatic carditis assumes importance and suggests that sensitisation may play a part in the rheumatic process. However until the specific rheumatic lesion is produced experimentally, and especially by bacterial sensitisation, the allergic hypothesis cannot be accepted without reserve.

The most interesting experimental work along these lines was carried out recently by Rich and Gregory (1943). Whilst engaged in the study of experimental polyarteritis nodosa these workers observed

cardiac lesions in the sensitised animal which they claimed showed many of the histological characters of the specific rheumatic granuloma. In view of the many previous but unsatisfactory claims of various workers and of the importance of Rich and Gregory's findings, it was considered desirable to verify and if possible extend their observations.

EXPERIMENTAL PROCEDURE

Eighteen rabbits were given an intravenous injection of 10 c.c. of horse serum per kg. body weight. A further eight animals received a similar amount by intraperitoneal injection. The animals were observed closely for the occurrence of cutaneous flushing. This was noted in the ears of the rabbits from the 12th to the 14th day and was accompanied by a rise in temperature. At this time a skin test was performed and found to be positive. On the 17th day following the first injection of serum the rabbits were given an intravenous injection of 1 c.c. of serum to absorb circulating antibody. Two days later the initial large dose of serum was repeated and all animals were killed one week later, that is, 26 days after the first serum injection. It is believed that this procedure establishes in the experimental animal conditions similar to those obtaining in serum sickness in man.

RESULTS

The vascular lesions to be described resulted after one injection of serum, but were more fulminant and widespread in the animals receiving the second large dose. The results were equally successful whether the intraperitoneal or intravenous route was used for the first injection. A study of the healing lesions was also made, the animals being killed at varying intervals after the second injection. In a further group of animals repeated injections were given at 4-weekly intervals over a period of 8 months.

The arterial lesions, which were present in 88 per cent. of the experimental animals, resembled closely those of polyarteritis nodosa. The vessels most frequently involved were those of the coronary system but sometimes there was an accompanying arteritis of the pulmonary, hepatic, renal and gastric vessels. The cardiac lesions are described first.

Coronary arteries. The whole coronary system was affected from the main vessels to the smallest arterioles. The first morphological change was œdema of the media, with swelling of the muscle fibres and loss of nuclear definition. The whole arterial wall became swollen and homogeneous and the lumen reduced in size. There was an accompanying perivascular œdema.

This was followed by inflammatory infiltration and fibrinoid necrosis of the vessel wall. Frequently the entire circumference was involved, but sometimes only a segment, or the inner or outer third. There was an accumulation of fibrin beneath the endothelium and the overlying endothelial cells became swollen and prominent.

As necrosis of the media proceeded it assumed a bright eosinophilic appearance and stained positively for fibrin. The periarterial in-

flammatory reaction which developed simultaneously was present even when the media showed hydropic change alone. Polymorphs and eosinophils appeared in large numbers in the adventitia. Sometimes they remained confined to this coat but frequently extended to the media and sub-endothelial layers (fig. 1). The polymorphs gradually gave way to large mononuclear cells. These surrounded and infiltrated the vessel and were accompanied by lymphocytes and mononuclears, which last showed the nuclear characters described by Anitschkow and were much larger than any of the other inflammatory cells. Their cytoplasm was basophilic and their nuclei were round or oval, with well marked nuclear membrane. Multinucleated forms were present (fig. 2). Lymphocytes, red cells and mononuclears, with a varying amount of clear fluid or fibrin, accumulated beneath the endothelium, but thrombosis was not observed nor did aneurysmal dilatation occur, although in many vessels the conditions necessary for its development seemed to be present. The acute phase was present from the 4th to the 12th day after the second injection of horse serum.

The inflammatory cells gradually disappeared and after the 14th day fibrinoid necrosis of the media was not conspicuous. Lymphocytes persisted in the adventitia and Anitschkow cells in the media. No vascularisation of the vessel was observed but healing took place by fibrous replacement of the necrotic muscle. The resulting scar was usually patchy in its distribution and was well demonstrated by means of stains for collagen. In such vessels there was sometimes an absence of muscle nuclei throughout the entire media. Frequently the intima was thickened by cellular connective tissue and the adventitia showed fibroblastic proliferation.

Finally complete healing occurred. Material for the study of this stage was provided by the series of animals which were killed at increasing intervals after the second serum injection. It was found that healing was generally established by the end of the first month after this date. The end result was extensive fibrosis of the coronary vessels with a varying degree of perivascular scarring depending on the extent of the periarterial lesion. Intimal fibrosis was often well developed. Even more marked were the arterial changes observed in animals which had been given repeated large doses of serum. Previous damage had resulted in almost complete medial fibrosis and thickening of the adventitia, while a superimposed acute inflammatory reaction indicated a more recent assault. The vascular lesions were accompanied by extensive myocardial fibrosis.

Myocardial Aschoff-like lesions. Small granulomatous nodules were frequently found in relation to the coronary arterioles and occasionally in the interstitial tissues of the myocardium without apparent relation to the blood vessels. Most often they occurred in the adventitia of the vessels, lying generally in the axis of the inter-stitial septa and extending distally along them. They appeared

concomitantly with the vascular lesions, but quite often were found in relation to a vessel which showed no damage to media or intima.

The nodule in its mature form bore a close resemblance to the Aschoff nodule of rheumatic fever and appeared to pass through a similar evolution (fig. 3). The first stage was the occurrence of small areas of paravascular necrosis of connective tissue. Sometimes these formed small discrete lesions well removed from the adventitia and surrounded by polymorphs which were eventually replaced by focal accumulations of large mononuclear and multinucleated cells of the Aschoff type, like those in the arterial lesions (fig. 4). However, the infiltration was not diffuse, the cells being grouped about a focus of swollen degenerated collagen fibrils and sometimes forming a rather palisade-like border (fig. 5). Lymphocytes, eosinophils and Anitschkow cells were present in the periphery of the nodule. In one instance the cells comprising the nodule underwent polarisation, becoming elongated and resembling closely a healing Aschoff nodule (fig. 6). In general, however, the end result was paravascular scarring, but not of the fibrillar type found in rheumatic fever.

Endocardium. Here again the earliest change was necrosis of collagen, individual fibres becoming swollen, eosinophilic and fragmented. The fully developed endocardial lesion was an Aschoff-like nodule, histologically identical with the myocardial lesion (fig. 7).

The second type of endocardial lesion closely resembled that described by Pappenheimer and VonGlahn (1927) in rheumatic endocarditis. Overlying a sub-endothelial band of necrotic collagen were palisades of proliferating cells, several layers deep, composed of lymphocytes, spindle-shaped cells, large mononuclears and polymorphs, replacing the surface endothelium and projecting into the lumen.

Thickening and fibrosis of the endocardium occurred as these acute lesions healed.

Valve ring. Lesions of the mitral valve, valve ring and angle were often present, even in the absence of myocardial changes. The aortic and tricuspid valves were similarly but less frequently involved. The pulmonary valve showed no specific lesion. The earliest finding was oedema of the connective tissue of the valve ring, followed by increased vascularity and diffuse inflammatory infiltration (fig. 8). In the more specific lesions large mononuclear cells of the Aschoff type were present, either diffusely or focally around damaged collagen. The character of the Aschoff cells was well defined in these ring lesions. Frequently each cell contained 4 or 5 nuclei arranged centrally, which were often so large as to fill the entire cell. The cytoplasm stained dark blue with haematoxylin and faded indefinitely into the background (fig. 9).

As the inflammatory reaction subsided, new capillaries appeared, and healing took place with the formation of a loose cellular fibrous tissue which later became hyalinised.

FIG. 1.—Rabbit: coronary artery, showing pan-arteritis. H and E ×70.

FIG. 2.—Arteritis, showing infiltration of vessel wall by lymphocytes and deeply staining large mononuclear cells, also perivascular inflammatory reaction with Aschoff nodule at each pole of vessel. H. and E. ×200.
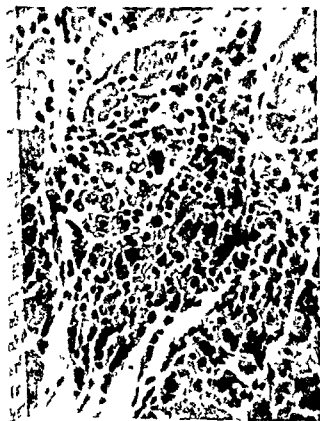
FIG. 3.—Myocardial nodule composed of aggregation of mono and multinucleated cells, with peripheral zone of lymphocytes. H. and E. ×290.

FIG. 4.—Paravascular nodule, showing focus of damaged collagen at one pole of vessel infiltrated by Aschoff cells. H. and E. ×175.

EXPERIMENTAL SERUM CARDITIS
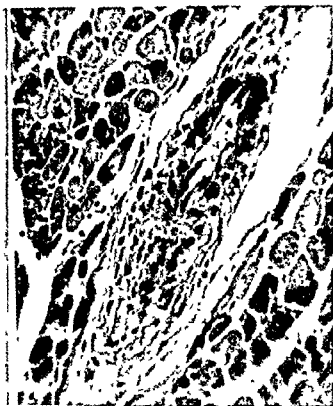
FIG 5 —Aschoff nodule, showing cells forming a palisade like border around a central zone of necrotic collagen fibrils. H. and E. ×275.
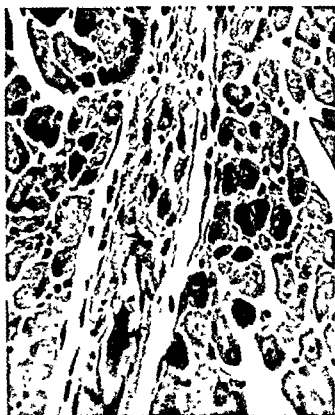
FIG 6 —Nodule showing polarisation, multinucleated cells becoming elongated and tending to lie in parallel lines. H. and E. ×275.
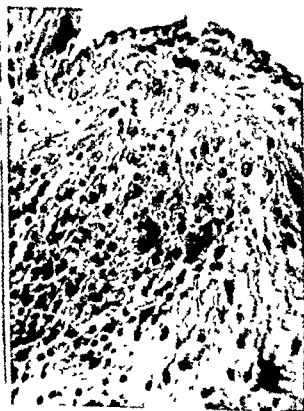
FIG 7 —Subendocardial Aschoff nodule, showing accumulation of characteristic cells Note proliferation of overlying endocardial cells. H. and E. ×260.

FIG. 8.—Valve ring note ed inflammatory infiltration with polymorphs, eosinophils and Anitschkow cells. There are two Aschoff nodules immediately adjacent to the swollen endocardial cells. H. and E. ×100.

Valve angle. Lesions in this position were usually associated with an underlying ring lesion. The endocardial cells became swollen and prominent, proliferation occurred and large surface projections of deeply basophilic spindle cells were formed. These often formed a palisade at right angles to the surface and for a time remained covered by intact endothelium (fig. 10). Areas of necrotic collagen were often seen between the proliferating cells. In the second type of valve-angle lesion, Aschoff-like nodes occurred just beneath the endocardium. Their presence here led to desquamation of the surface endothelium and was associated with a diffuse inflammatory infiltration.

Valve. In the early stages the valve leaflet became swollen and diffusely infiltrated with polymorphs, Anitschkow cells, lymphocytes and eosinophils. Connective tissue cells appeared swollen and the ground substance assumed a granular appearance. Necrosis of collagen also occurred, usually in the fibrosa and auricular layer of the spongiosa. The characteristic Aschoff-like cells accumulated in nodule formation and vascularisation of the valve was observed, the small capillaries being accompanied by non-specific inflammatory cells (fig. 11). When the nodule was more closely related to the surface of the valve, proliferative changes were observed in the overlying endocardium, the endothelium becoming elevated by large numbers of inflammatory and large mononuclear cells arranged in a palisade at right angles to the underlying zone of damaged collagen (fig. 12).

When necrosis of collagen involved the surface of the valve, the endothelium was replaced by bright eosinophilic material in the base of which were large mononuclear cells of Aschoff type (fig. 13). Occasionally nodular vegetations formed which projected from the surface and were covered by a layer of necrotic endocardium. Deposition of platelet thrombi on these lesions of the valve surface was not observed. Healing of the valvular lesions occurred, with vascularisation and fibrosis, and this was most marked in the animals which had received repeated injections of serum.

Aorta. Intimal proliferation of cells was frequently observed. The sub-endothelial tissues were diffusely infiltrated by lymphocytes and polymorphs. Occasionally the endothelial cells appeared to proliferate, becoming multinucleated and deeply basophilic and projecting above the surface. Intimal change was usually secondary to eosinophilic swelling of the underlying collagen fibrils. As healing occurred, intimal fibrosis was observed. No gross lesion was noted in the media apart from hydropic swelling of the muscle fibres. The adventitia and vasa vasorum were normal.

Vascular lesions in the viscera

Lungs. The vessels here occasionally showed periarterial inflammatory infiltration with polymorphs and eosinophils. Similar cells were also observed in the media and occasionally there was

sub-endothelial accumulation of lymphocytes, monocytes and red cells, while the underlying media was hydropic (fig. 14). Fibrinoid necrosis of the vessels, however, was not observed.

Liver. The vascular lesions were identical with those occurring in polyarteritis nodosa. In one animal almost every artery in the portal tracts was affected and every stage of the process was observed. There was early fibrinoid necrosis of the inner or outer third of the media, with or without an inflammatory reaction, which, when present, consisted in an infiltration of all coats by polymorphs, lymphocytes and eosinophils (fig. 15). This was accompanied by proliferation of the sub-endothelial tissues, with occlusion of the vessel by this mechanism or by thrombosis. In lesions of longer standing the inflammatory cells were chiefly mononuclear and healing took place by fibroblastic proliferation. Eventually the necrotic media was replaced by connective tissue. Re-canalisation of occluded vessels was seen and in the completely healed lesion there was extensive fibrosis of the portal tracts, radiating outwards from the adventitia of the affected blood vessels.

Spleen. Vascular lesions of polyarteritis nodosa type were encountered in the central arterioles of the Malpighian bodies. The vessel wall did not undergo actual necrosis but the muscle cells became swollen and their nuclei prominent. The adventitia was infiltrated by polymorphs, eosinophils and mononuclear cells, which stood out in contrast with the lymphocytes of the adjacent Malpighian body. The lesion was a true periarteriolitis, and as healing occurred periarteriolar fibrosis resulted.

The lesions thus far described are representative of those found elsewhere in the experimental animal and a detailed description of these is unnecessary. It is sufficient to state that among other vessels occasionally involved were the gastric, vesical and uterine arterioles.

DISCUSSION

These results show that sensitisation of rabbits by a bland non-toxic protein can produce a series of lesions confined to the cardio-vascular system and that such lesions have a specific histological structure and a specific distribution. In the heart the earliest lesion appears to be a focal necrosis of collagen. These focal necroses are found in the paravascular tissues, the interstitial septa of the myocardium and the fibrous tissue of the valve ring and valves themselves. Associated with this necrosis there develops an inflammatory reaction composed, in the acute phase, of polymorphs and eosinophil leucocytes, which are eventually replaced by large mononuclears of peculiar character and by Anitschkow cells. Whilst it is of much interest that sensitisation produces a lesion of this type and distribution, the important problem is to determine whether or not this serum lesion is indeed a reduplication of the rheumatic granuloma.

In a previous paper (McKeown, 1945) the evolution of the rheumatic lesion has been studied in the human material available in this Institute. The primary lesion appears to be a focal necrosis of collagen, followed by emigration of polymorphonuclears and eosinophils. These soon give way to a focal aggregation of large mononuclears, some of which may become bi- or multinucleated. Such lesions are found in the valves, valve rings, endocardium and interstitial septa of the myocardium. The necrosis of collagen in these specific sites is of the greatest importance in determining the subsequent evolution of the rheumatic process, and the variation in the histological characters of the fully mature lesion is due, not to alteration in the tissue response, but to certain modifications dictated by the site of the lesion.

In the experimental animal, lesions which bear a striking resemblance to the Aschoff nodule are present in the paravascular tissues in the myocardium. The large mononuclear cells infiltrating and surrounding the focus of necrotic collagen are similar to those of the Aschoff nodule. Their cytoplasm stains red with methyl green-pyronin, their nuclei are characteristic and occasionally multinucleated forms occur. Nuclei with the characters of the Anitschkow cell nucleus are also present. Hence in histological structure and in site the experimental lesion is identical with the specific granuloma of the naturally occurring disease.

The analogy is further supported by the occurrence of endocardial and valvular lesions of similar nature. All degrees of valvulitis are present in the experimental animals and in some this is the only manifestation of a serum lesion in the heart. Whilst sections of all four valves were examined, the serum lesion was found most frequently in the mitral valve and in no case was it observed in a pulmonary leaflet. Where the necrosis of collagen is deep in the substance of the valve the cellular infiltration and subsequent evolution of the lesion are identical with those seen in rheumatic fever. When the necrosis is more superficial the necrotic collagen appears to be extruded on to the surface of the valve as a vegetation, or to stimulate a marked proliferation of the overlying endocardial and immediately sub-endocardial cells so that there is a close resemblance to the palisading of cells so often seen in the human valve. As in rheumatic fever there is involvement of the valve ring and in the mitral of the valve angle also. Analysis of these valvular lesions shows their close identity with those of rheumatic valvulitis—the frequent involvement of the mitral valve, the focal nature of the mitral lesion and the similarity in type of reaction. Every stage of the whole process finds its parallel in the evolution of rheumatic valvular disease. Even endocardial lesions other than valvular were most marked in the posterior wall of the left auricle.

Finally, a study of the coronary arteries showed that these are implicated in precisely the same manner in the experimental animal

as in rheumatic fever. Fibrinoid necrosis of the media with an acute inflammatory infiltration of all coats is frequently observed. Even in their more specific characters the vessel lesions are identical, large mononuclears of Aschoff type infiltrating the adventitia or occurring within the vessel wall. As healing occurs, medial fibrosis develops as in healed rheumatic arteritis. These analogies in site and quality of the lesions strongly suggest that hypersensitivity is the essential mechanism in the production of the rheumatic lesion.

This leads to a consideration of the possible factor responsible for sensitisation in acute rheumatism—its nature, origin and mode of action. At present we can only surmise that it is probably the product of micro-organisms. Lichtwitz (1944, p. 32) believes that in most instances " a sort of bacterial fifth column enters the body, finds lodgement in an unsuspecting place such as a sinus or tonsil, and from that focus sends out antigens which disorganise defence and create sensitization ". An organism which may play an important part in this role is the hæmolytic streptococcus, in view of the frequent occurrence of streptococcal infections of the upper respiratory tract preceding an attack of acute rheumatic fever. This view is strongly supported by the work of Coburn (1931), who has shown that during an attack of acute rheumatism the patient is hypersensitive in an anaphylactic manner to the products of the hæmolytic streptococcus. In the course of this experimental work attempts have been made to sensitise animals with large intravenous doses of killed hæmolytic streptococci, so far without success. So, although most clinical and serological observations tend to incriminate the hæmolytic strepto-coccus, complete proof is still lacking.

There are always two aspects which require consideration in any disease process, first the ætiological agent concerned, and second the reaction of the body to its presence. In other words, the specificity of the rheumatic response may depend not entirely on the character of the infecting organism but perhaps also on some individual reaction of the rheumatic subject, or some peculiarity of the vascular mesenchyme. Such a conception would help to explain the characteristic distribution and nature of the rheumatic process on an anaphylactic basis. But before the anaphylactic mechanism can be accepted as the essential factor in the production of the rheumatic state, its exact relationship to the rheumatic process and the importance of micro-organisms as the probable means of sensitisation will require further elucidation.

CONCLUSIONS

From these experiments it seems justifiable to draw the following conclusions :—

1. Lesions with the fundamental characteristics of rheumatic fever have been produced in the serum-sensitised animal, thus

EXPERIMENTAL SERUM CARDITIS
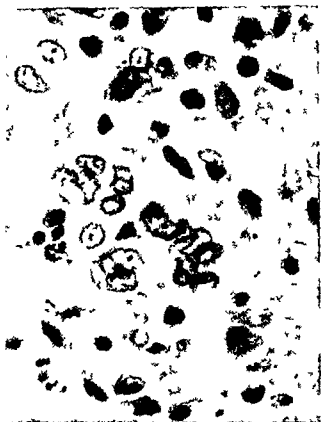
 9 —Valve ring Aschoff nodule showing
several multinucleated Aschoff lik e cells The
nuclei are large, arranged centrally and have
a well defined nuclear membrane H and E
×700

FIG 10 —Valve angle proliferative lesion,
showing palisading of cells with endocardial
cells still intact on surface of projection H
and E ×250
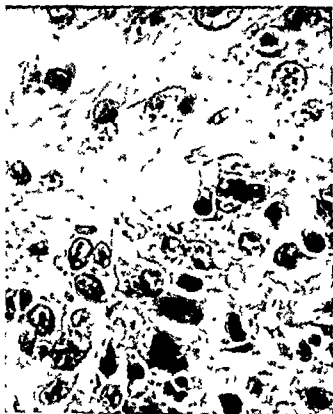
IG 11 —Aschoff nodule in valve composed of
characteristic multinucleated cells H and F
×650

FIG 12 —Valve proliferative lesion on endo
cardial surface, cells forming a palisade to an
underlying lesion of the valve substance which
shows collagen necrosis and cellular infiltra
tion H and E ×390

EXPERIMENTAL SERUM CARDITIS
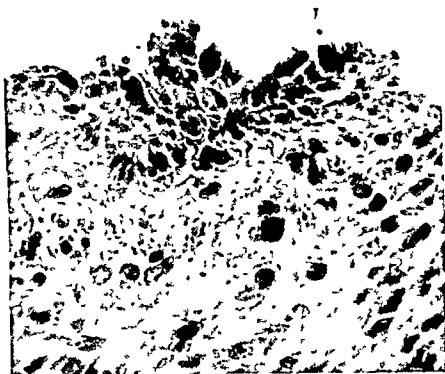
FIG 13—Valve surface necrosis of collagen with proliferation of Aschoff like cells in base of lesion   H and E   ×425

FIG 14—Lung vessel showing arteritis involving chiefly the sub endothelial zone, with accumulation of fluid, lymphocytes, mono nuclears and red cells beneath the endothelium H and E   ×130
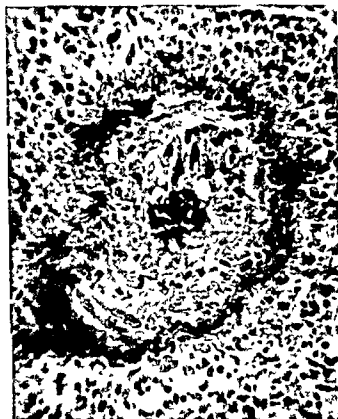
FIG 15—Liver vessel in portal tract showing fibrinoid necrosis of outer third of media, associated with sub endothelial proliferation and an acute inflammatory infiltration of the adventitia   H and E   ×225

supporting the theory that rheumatic fever may be the result of hypersensitivity of the anaphylactic type.

2. While there is no clear indication as to the causal factor responsible for sensitisation, it is suggested on clinical grounds that the hæmolytic streptococcus is more closely allied to rheumatic fever than any other organism and that hypersensitivity is probably the result of bacterial sensitisation, possibly of streptococcal origin.

REFERENCES

ASCHOFF, L. . . . . 1935. *Med. Klin.*, xxxi, 1.
CLARK, E., AND KAPLAN, B. I. . 1937. *Arch. Path.*, xxiv, 458.
COBURN, A. F. . . . . . . 1931. The factor of infection in the rheumatic state, *London*.
FRIEDBERG, C. K., AND GROSS, L. 1934. *Arch. Int. Med.*, liv, 170.
JUNGHANS, E. . . . . . . 1933-34. *Beitr. path. Anat.*, xcii, 467.
KLINGE, F. . . . . 1933. Der Rheumatismus, *Munich*.
LICHTWITZ, L. . . . . . . 1944. Pathology and therapy of rheumatic fever, ed. by William Chester, *New York*, p. 32.
MCKEOWN, E. FLORENCE . . . 1945. *Ulster Med. J.*, xiv, 97.
MENZER . . . . 1902. *Berl. klin. Wschr.*, xxxix, 10 and 32.
PAPPENHEIMER, A. M., AND VON- 1927. *Amer. J. Path.*, iii, 583.
  GLAHN, W. C.
RICH, A. R. . . . . 1945. *Bull. Johns Hopkins Hosp.*, lxxvii, 43.
RICH, A. R., AND GREGORY, J. E. 1943. *Ibid.*, lxxii, 65.
VAUBEL, E. . . . . 1932. *Beitr. path. Anat.*, lxxxix, 374.

THE RELATIONSHIP BETWEEN RHEUMATIC CARDITIS AND SUBACUTE BACTERIAL ENDO-CARDITIS

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(PLATES LXXVIII AND LXXIX)

FROM the early years of the twentieth century onwards a steady stream of work has appeared emphasising strongly the frequent association and ætiological relationships of rheumatic and bacterial endocarditis, particularly the subacute type.

Clinically the number of cases of subacute bacterial endocarditis giving a previous history of diseases of the rheumatic group has been estimated by various authors at from 30 to 100 per cent. Pathologically it is almost axiomatic that valves which are the site of bacterial vegetations of the subacute type have suffered previous damage, generally rheumatic in nature. Hadfield and Garrod (1942) say that most authors have found this to be true in over 80 per cent. of cases. In other cases the original lesion has been a congenital defect or, more rarely, syphilitic or atherosclerotic valvular disease.

The important question is the mechanism by which the diseased valve favours the deposition of organisms. The available evidence suggests that primary bacterial localisation occurs on a prepared focus on the endocardial surface, and that vascular embolism plays no part. Allen (1939) points out that, in valves previously damaged, mechanical factors are of importance in determining the site of implantation.

The nature of the surface changes, the mechanism of their production and in particular their relation to rheumatic fever, are all problems which require further elucidation.

In any attempted evaluation of the relationship between rheumatic fever and subacute bacterial endocarditis it seems imperative to attempt to estimate the duration of the lesions in each of these diseases. This is often difficult on clinical data alone, as in the one case the illness is so often sub-clinical in its manifestations, and in the other so insidious in its onset. Both conditions cause structural alterations in the heart, and there is evidence to suggest that, at least as far as the rheumatic lesions are concerned, the histological appearances may be indicative of the length of time they have been present.

Aschoff (1905) was the first to suggest that the lesion which now bears his name is specific for rheumatic fever. He also anticipated future descriptions of a typical life cycle by suggesting the ultimate transformation of this lesion into connective tissue. A similar view was expressed by Geipel (1905). Talalajew (1929) described three phases in the progression of the lesion—swelling and exudation, proliferation, and finally sclerosis. Klinge (1933) also described a characteristic evolution of the lesion, with appearances typical of each stage of development.

In 1934 Gross and Ehrlich presented a histological classification of the myocardial Aschoff body based on a study of 70 hearts. In every case it was considered that diseases other than rheumatism could be excluded. They stated that during the first four weeks the lesion is non-specific and consists of swelling and fusion of the interstitial collagen fibres; later there is exudation of cells indistinguishable from lymphocytes.

From the 4th to the 9th week the lesions are said to be characterised by the presence of round or oval cells, larger than lymphocytes. These cells have a delicate mantle of basophilic cytoplasm with a clearly defined edge. The nuclei are of the type described as common to all forms of the Aschoff body—fibro-cytoid, owl-eyed, or pyknotic. Giant cells are occasionally seen. There may be a peripheral zone of lymphocytes and neutrophil polymorphs.

The middle phases of evolution they believe to extend from the 9th to perhaps the 16th week. Giant cells are frequent. They may contain seven or more nuclei irregularly arranged at the centre of the cell. These giant cells may assume the form of large syncitial basophilic masses, with ragged cytoplasm. Towards the end of this period the cells gradually elongate to a spindle form and the cytoplasm becomes less obvious. Simultaneously the whole lesion assumes a definite orientation within the planes of the myocardial septa, giving rise to the " polarised " type of lesion. This stage is believed to represent an intermediate phase in the metamorphosis of the cells into fibroblasts.

The fibrillar variety of Aschoff node may be seen from the 13th week onwards. The scanty cytoplasm is represented by rather blunt basophilic knobs at either end of the attenuated cell. The nuclei are largely fibrocytoid. Giant cells are infrequent. The collagen is seen as fibrillar strands. This is the stage which precedes the complete transition of the cells to fibroblasts.

Gross and Ehrlich found a remarkable consistency in the structure, and therefore presumably in the age, of the nodules seen in any individual section. They therefore assumed that a given crop of lesions can be timed from the onset of a given attack of rheumatic fever. Since almost all the nodules reach the same evolutionary stage in development, as judged by their appearance at the time of death, it seems that they pass through an orderly and consistent sequence of changes. In cases in which the onset of the rheumatic attack is known, the duration corresponds to the estimated morphological age of the myocardial Aschoff body.

McKeown (1945) studied the clinical and post-mortem findings in 36 cases of rheumatic fever. She concluded that it is possible both to trace the evolutionary changes occurring in the Aschoff node and to estimate its age. In general, her findings are in agreement with those of Gross and Ehrlich.

It thus appears that there is a reliable histological method of diagnosing not only the presence of rheumatic carditis but also its duration, and it is considered that this might in turn provide an opportunity of clarifying the relationship between this disease and subacute bacterial endocarditis. With this object in mind, a study was made of 34 cases of subacute and 12 cases of acute bacterial endocarditis.

EVOLUTION OF THE RHEUMATIC LESION IN CASES OF
SUBACUTE BACTERIAL ENDOCARDITIS

FIG. 1.—A. 2213 Myocardial Aschoff nodule showing necrosis of collagen and the presence of mononuclear and giant cells. There are a few lymphocytes towards the periphery of the lesion. Approximate age of lesion 2 months. ×140.
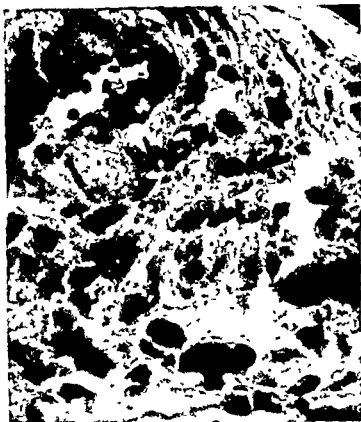
FIG. 2.—A. 2519. Acute rheumatic lesion beside coronary arteriole. Note necrotic collagen and presence of giant cells and Anitschkow cells with "owl eye" nuclei. The vessel wall is swollen and œdematous. ×475.
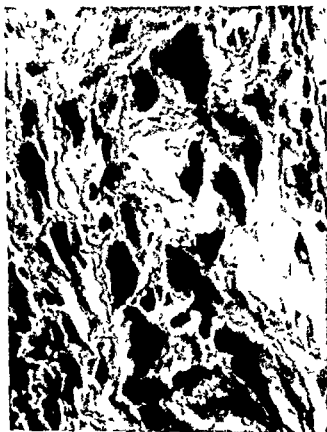
FIG. 3.—A 3128 High power view of acute Aschoff node. There is necrosis of collagen, and several multi-nucleated giant cells with deeply staining cytoplasm are present. ×550.

FIG. 4.—A. 2126. Myocardial Aschoff lesion showing early polarisation. Much of the necrotic collagen has been absorbed. Giant cells are still present. Approximate age of lesion 2-4 months. ×190.

Material and methods

In 8 of the subacute cases the whole heart was available for study. Sections from these were cut according to the method of Gross, Antopol and Sacks (1930). In the remainder the routine autopsy blocks were studied. All sections were stained by hæmatoxylin and eosin and by Masson's trichrome stain. Where necessary in individual instances additional stains were used.

Results

In every case in which Aschoff nodes were present an attempt was made to estimate their age on the basis of the histological appearances. For purposes of tabulation the lesions were divided into four age-groups in accordance with the stages of evolution already described.

1. *Under two months* (figs. 1-3). Exudative stage, characterised by necrosis of collagen and aggregation of large mononuclear cells. Giant cells are sometimes seen. In the first half of this period non-specific inflammatory cells are frequently present, specially around the periphery.

2. *Two to four months* (figs. 4 and 5). Proliferative phase. Giant cells are generally present in the earlier part of this period, later becoming more oval in shape and showing less cytoplasm. There is also elongation of individual cells and polarisation of the whole nodule.

3. *Four to six months* (figs. 6 and 7). Fibrillar stage, characterised by retrogressive fibroblastic changes. Gradual diminution in the cytoplasm of the cells is noted. Fine reticular bands of fibrous tissue are formed. The "Aschoff" nature of some of the cells is still apparent. The nuclei are mainly fibrocytoid. Staining by the Unna-Pappenheim method frequently shows the presence of cells with red cytoplasm, such as are frequently seen in the more acute lesions.

4. *Over 6 months* (fig. 8). Sclerotic phase, in which the typical feature is the laying down of parallel bands of dense fibrous tissue. The lesions are relatively acellular, and become increasingly so with the passage of time. No further evolution occurs and the ultimate lesion —a fairly dense fibrous scar usually para-vascular in position— cannot be dated beyond this stage with any accuracy. There is no essential difference between the Aschoff nodules observed in these cases and those described by other authors in cases of uncomplicated rheumatic carditis.

In addition, many of the cases present vascular changes which, on the basis of reports in the literature, are considered to represent a rheumatic arteritis. Similar arterial lesions have been described in detail by McKeown. It is considered that these specific vascular alterations are additional evidence in favour of the rheumatic nature of the myocardial lesions. In many of the hearts, crops of rheumatic lesions of two or even three different age-periods are present. In the 34 cases of subacute bacterial endocarditis, evidence of active arteritis is present in 38 per cent. and healed arteritis in 32 per cent. Altogether

active rheumatic stigmata of various types are present in 79 per cent. and healed lesions in 91 per cent.

The incidence and estimated age of the myocardial Aschoff bodies in these cases and in the 12 acute cases are shown in tables I and II.

TABLE I

Incidence and age of Aschoff nodules in subacute bacterial endocarditis

Case no.	Under 2 months	2-4 months	4-6 months	Over 6 months
A. 2126	+	+	−	+
A. 2142	−	+	+	+
A. 2213	−	+	+	+
A. 2319	−	−	+	+
A. 2305	−	−	−	−
A. 2345	−	−	+	+
A. 2436	−	+	−	+
A. 2066	−	−	+	−
A. 2669	−	−	+	+
A. 4047	−	+	−	+
A. 2674	−	−	+	+
A. 3128	−	+	+	+
A. 2917	−	−	+	+
BA. 3	−	−	+	+
A. 3784	−	−	−	+
A. 2703	−	−	+	+
A. 3717	−	−	+	+
BA. 22	+	−	−	+
BA. 82	−	−	−	−
A. 2704	−	+	−	−
A. 3380	−	−	−	+
A. 4089	−	−	+	+
A. 2376	−	−	−	−
A. 2764	+	−	−	−
A. 2987	−	−	+	−
A. 3069	−	−	+	+
L. 1	−	−	+	−
L. 2	−	−	+	+
L. 3	−	−	+	−
L. 4	−	−	−	−
L. 5	+	−	−	+
L. 6	+	−	+	+
A. 4263	−	−	+	+
A. 4341	−	−	+	+
Total . . .	4	7	20	23

The most striking fact emerging from these results is that in the subacute cases, in which the average duration is thought to be about six months, 26 (76 per cent.) show rheumatic lesions interpreted as being of six months' duration or less. In the acute variety, which is characterised by its much shorter course, 3/12 (25 per cent.) of the hearts show Aschoff bodies of less than two months' duration. In 5/12 (42 per cent.) the myocardial lesions appear to be of less than four months' duration.

In view of these results an attempt was made to correlate the morphological findings with the approximate clinical duration of the

EVOLUTION OF THE RHEUMATIC LESION IN CASES OF
SUBACUTE BACTERIAL ENDOCARDITIS
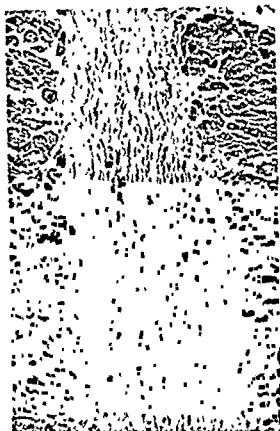
FIG. 5.—A. 2437. Later stage of polarisation of Aschoff nodule. The individual cells have become spindle-shaped and the whole lesion has been *orientated so that the long axis runs* parallel with that of the myocardial septum. Approximate age of lesion 4 months. ×120.

FIG. 6.—A. 2126. *Higher-power* view of polarising lesion to show the elongation of the cells, the *decrease in cytoplasm*, and the laying down of new collagen strands which later results in *paravascular fibrous scarring.* ×190.
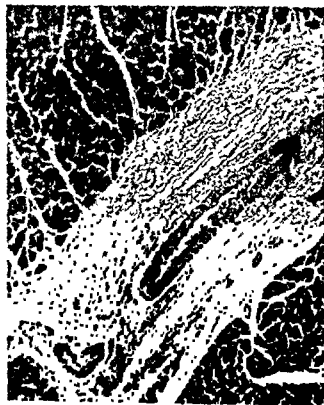
FIG. 7.—A. 4263. *Healed rheumatic para-vascular scarring.* The tissue is still fairly cellular and is probably of about 6 months' duration. ×99.

FIG. 8.—A. 2269. Fibrillar "onion-skin" para-vascular scarring, regarded as the ultimate result of healing of the Aschoff nodule. ×275.

bacterial endocarditis. The latter is of course extremely difficult to estimate with accuracy, and for this reason some cases have been

TABLE II

Incidence and age of Aschoff bodies in acute endocarditis

Case no	Under 2 months	2-4 months	4-6 months	Over 6 months
A. 3829	—	+	—	—
A. 3313	—	—	—	—
A. 3556	—	—	+	—
A. 3834	—	—	+	+
A. 2519	+	+	—	—
A. 2482	—	—	—	—
A. 2766	—	+	—	+
A. 3174	+	+	—	—
A. 3698	—	—	—	—
A. 2751	—	—	—	—
A. 3265	—	—	—	+
A. 2423	+	—	—	+
Total . . .	3	4	1	4

omitted from the next table. The onset was taken as being coincident with the first definite complaint of malaise made by the patient. The results are shown in table III. Of the 17 subacute cases in which it is possible to make an approximation of the clinical duration, Aschoff bodies of a corresponding age are seen in 11 (64 per cent.). In the acute cases the corresponding figures are 2/8 (25 per cent.).

Discussion

Although very few observers have attempted to correlate the duration of the two types of lesion, this finding of Aschoff bodies in the myocardium of patients with infective endocarditis has been noted in many of the reports in the literature. Among these are the figures shown in table IV.

VonGlahn and Pappenheimer (1935) make the generalisation that Aschoff nodules are found in the myocardium of practically the same proportion of cases of subacute bacterial endocarditis as of uncomplicated rheumatic disease. In this connection it is desired to emphasise that the examination of only one routine block is insufficient for the exclusion of rheumatic stigmata.

The importance to be attached to this finding of recent Aschoff bodies in cases of bacterial endocarditis depends on its interpretation. The essential point is whether or not one is justified in considering the lesions specific evidence of rheumatic carditis. The reasons generally advanced in favour of specificity are that the nodules have distinctive histological characters. They are frequently seen in acute rheumatism and infrequently in other acute infectious diseases.

Coombs (1908-09) states that these lesions appear to be characteristic of rheumatic as opposed to other forms of carditis, and that changes

TABLE III

Correlation of clinical duration with presence and age of Aschoff bodies

Subacute bacterial endocarditis		
Case no.	Approximate clinical duration	Presence and age of Aschoff bodies
A. 3128	4 months	2-4 months, 4-6 months
A. 3069	5 ,,	None seen, but valve showed active rheumatic valvulitis
BA. 3	3-4 ,,	4-6 months
A. 2703	6 ,,	4-6 ,,
A. 3717	6 ,,	4-6 ,,
BA. 22	7 ,,	Under 2 months, and old lesions
A. 4089	3 ,,	4-6 months
A. 4047	4 ,,	2-4 ,,
A. 2376	1 month	Old
A. 2764	Several months	Under 2 months
A. 2345	3 months	4-6 months
A. 2319	6 ,,	4-6 ,,
A. 2917	8 ,,	4-6 ,,
A. 2142	6 ,,	2-4 months, 4-6 months
A. 2669	4 ,,	4-6 ,,
L. 4	4 ,,	None seen
A. 4263	6 ,,	4-6 months
Acute cases		
A. 2423	4 weeks	Under 2 months
A. 2766	6 ,,	,, 2 ,,
A. 2751	4 ,,	None seen
A. 3265	4 ,,	,, ,,
A. 3829	1 week	4-5 months
A. 3313	1 ,,	None seen
A. 3556	6 weeks	,, ,,
A. 2482	2 months	,, ,,

TABLE IV

Incidence of Aschoff bodies in subacute bacterial endocarditis
(various published series)

Authors	No. of cases subacute endocarditis	Percentage with Aschoff bodies
Clawson and Bell (1926)	61	11·5
Gross and Fried (1937)	30	30·0
Buchbinder and Saphir (1939) . . .	40	37·5
Saphir (1935)	35	40·0
Clawson (1929)	60	45·0
VonGlahn and Pappenheimer (1935) . .	26	46·0
Saphir and Wile (1933-34) . . .	10	100·0

histologically similar are seen in rheumatic endocarditis and pericarditis and in subcutaneous rheumatic nodules.

Gross and Fried (1937) are of the opinion that implantation of bacteria such as occurs in ulcerative endocarditis may lead to reactivation of a smouldering rheumatic infection. They consider that this view, which is also expressed by Graybiel and White (1936), affords an explanation of the high incidence of Aschoff bodies in cases of superimposed endocarditis. They offer no explanation as to how or why this reactivation occurs in bacterial endocarditis, or why it does not occur in other infectious conditions, in which Aschoff bodies are rarely found.

A somewhat similar view is held by those who find in streptococcal infection the aetiological agent of rheumatic fever. The suggestion made in this case is that the streptococci have been responsible for both lesions—that in fact the two conditions are but different manifestations of the same process. This idea is also held by Clawson and Bell (1926).

There are several facts which appear to render this hypothesis untenable. First, it is the *Streptococcus haemolyticus* which is generally considered to be important in the aetiology of rheumatic fever, and there is no evidence to incriminate members of the *viridans* group. The latter are however the organisms most commonly found in cases of subacute bacterial endocarditis. Further, in the material forming the basis of the present paper, Aschoff nodules were found in three cases of pneumococcal endocarditis, and in two other cases, one due to a staphylococcus, the other to *Haemophilus para-influenzae*. The literature contains scattered reports of endocarditis due to *Brucella abortus* and other organisms in which Aschoff bodies were present. Hitzig and Liebesman (1944) report the occurrence of an endocarditis due to *Spirillum minus* in association with typical rheumatic lesions in the myocardium. Finally, in cases where the bacterial infection is superimposed on a true congenitally malformed heart, Aschoff bodies are often absent, though the infective process is presumably the same. For these reasons, the suggestion that the Aschoff bodies in cases of subacute bacterial endocarditis represent either a specific or a non-specific reaction to the bacterial infection cannot be considered valid.

Held and Lieberson (1943) suggest that the vegetation of bacterial endocarditis, with its exuded fibrin, blood platelets and enmeshed bacteria, is a manifestation of a high general and local tissue immunity to bacterial invasion. In a discussion on the same point in 1935, Saphir states that the finding of typical Aschoff bodies in the myocardium in cases of subacute bacterial endocarditis may be taken as evidence against the assumption that the latter is an immune response in a previously hypersensitive patient. He felt that it would be difficult to explain why a tissue should respond simultaneously in two different ways—for example with a hypersensitive reaction, of which he considers the Aschoff body to be a type, and by an immune reaction, namely the vegetation seen on the endocardium.

Hence it would appear that subacute bacterial endocarditis can hardly be held responsible for the presence of myocardial Aschoff bodies and it may justifiably be concluded that these lesions are specific evidence of a rheumatic carditis.

If this is so, the finding of recent rheumatic lesions in cases of bacterial endocarditis becomes a factor of ætiological significance in the development of the latter disease. In cases where detailed clinical histories were available the estimated morphological age of the Aschoff bodies corresponded to the approximate clinical duration of the bacterial endocarditis in 64·7 per cent. of the subacute and in 25 per cent. of the acute cases. This high degree of correlation in the apparent duration of the two diseases strongly suggests that at the very moment of implantation of the bacteria the heart was the site of acute rheumatic lesions.

A similar view was expressed in 1935 by both Saphir and VonGlahn and Pappenheimer. The latter authors also point out that the engrafting on rheumatic hearts of vegetations due to infection by organisms other than streptococci is a cogent argument against the view that the two types of lesion are a response of different intensity to the same infective agent, unless one dispenses with current views as to the histological specificity of the rheumatic reaction.

Clinical features which support the current hypothesis are that subacute bacterial vegetations are commonest on the valves which are most liable to damage by rheumatic fever and that they are also frequent on the posterior wall of the left auricle, where rheumatic stigmata are often found. The patient most likely to develop bacterial endo-carditis is the young adult with a well-compensated valvular lesion. This is interpreted as being due to the fact that at this age recurrent rheumatic attacks, even though often sub-clinical in their manifesta-tions, are frequent. As age advances the tendency to recurrence diminishes.

In a previous paper (MacIlwaine, 1945) the valvular changes occurring during an acute exacerbation of rheumatic fever were described, and it was shown that these are such as to offer a focus for the localisation of bacteria from the circulating blood. It was suggested that the initial stage in the development of bacterial endocarditis is the implantation of organisms on unhealed rheumatic verrucæ. Microphotographs illustrating this process were shown.

In the acute cases the necessity for a prepared focus decreases, due to the virulence and invasiveness of the organisms concerned. In subacute bacterial endocarditis the organisms are relatively avirulent and require for their implantation some localising factors in the valves themselves.

The purpose of this paper is to suggest that such a factor is the presence of acute rheumatic lesions, and that these are the determining pathogenic factor in the majority of cases of subacute and in a smaller percentage of cases of acute bacterial endocarditis.

Summary

1. Evidence is presented to indicate that the myocardial Aschoff nodule is a specific reaction of the tissues to an attack of rheumatic fever.

2. This lesion appears to evolve through an orderly and characteristic process of ageing. The appearances at any given time are diagnostic of the phase reached in this process.

3. A study of 34 cases of subacute and of 12 cases of acute bacterial endocarditis indicated that a large number showed evidence of rheumatic arteritis. Of more importance was the finding, in an even higher percentage, of Aschoff nodules of an age which corresponded to the clinical duration of the endocarditis.

4. The conclusion has therefore been reached that in many cases of endocarditis lenta, and in a smaller number of cases of acute bacterial endocarditis, the bacterial lesion is superimposed upon a heart which is, at the time of infection, the site of an active rheumatic carditis.

REFERENCES

ALLEN, A. C.	1939.	*Arch. Path.*, xxvii, 399.
ASCHOFF, L.	1905.	*Verhandl. d. dtsch. path. Gesellsch.* (1904), viii, 46.
BUCHBINDER, W. C., AND SAPHIR, O.	1939.	*Arch. Int. Med.*, lxiv, 336.
CLAWSON, B. J.	1929.	*Arch. Path.*, viii, 664.
CLAWSON, B. J., AND BELL, E. T.	1926.	*Arch. Int. Med.*, xxxvii, 66.
COOMBS, C.	1908-09.	*Quart. J. Med.*, ii, 26.
GEIPEL, P.	1905.	*Dtsch. Arch. klin. Med.*, lxxxv, 75.
GRAYDIEL, A., AND WHITE, P. D.	1936.	*Arch. Int. Med.*, lvii, 791.
GROSS, L., ANTOPOL, W., AND SACKS, B.	1930.	*Arch. Path.*, x, 840.
GROSS, L., AND EHRLICH, J. C.	1934.	*Amer. J. Path.*, x., 467.
GROSS, L., AND FRIED, B. M.	1937.	*Ibid.*, xiii, 769.
HADFIELD, G., AND GARROD, L. P.	1942.	*Recent advances in pathology*, 4th ed., *London*, p. 164.
HELD, I. W., AND LIEBERSON, A.	1943.	*Amer. Heart J.*, xxv, 478.
HITZIG, W. M., AND LIEBESMAN, ADA	1944.	*Arch. Int. Med.*, lxxiii, 415.
KLINGE, F.	1933.	Der Rheumatismus, *Munich*.
MacILWAINE, YVONNE	1945.	*Ulster Med. J.*, xiv, 108.
McKEOWN, E. FLORENCE	1945.	*Ibid.*, xiv, 97.
SAPHIR, O.	1935.	*Amer. J. Path.*, xi, 143.
SAPHIR, O., AND WILE, S. A.	1933-34.	*Amer. Heart J.*, ix, 29.
TALALAJEW, W. T.	1929.	*Klin. Wschr.*, viii, 124.
VONGLAHN, W. C., AND PAPPENHEIMER, A. M.	1935.	*Arch. Int. Med.*, lv, 173.

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THE NATURE AND SIGNIFICANCE OF THE CEMENTING SUBSTANCE IN INTERSTITIAL CONNECTIVE TISSUE

T. D. DAY

From the Department of Pathology, St Thomas's Hospital Medical School, London

(PLATES LXXX AND LXXXI)

IN 1876 W. Flemming described a cementing substance by which the fibrils composing the bundles of loose connective tissue were held together. He showed that in weak acids there was coagulation of this substance in contrast to the fibrils, which became swollen. The constricting rings seen in places along bundles swollen in acid he ascribed to irregular deposits of the cement substance, the irregularity of its distribution being partly caused by technical manipulations. The present communication is mainly concerned with a re-statement and amplification of these views.

MATERIAL AND METHODS

The material studied was the deep fascia covering the thigh muscles of the rat. The rats used came from a pure inbred strain maintained for another purpose in this laboratory. They were killed by a brisk blow on the head and pinned out. In order to avoid drying, the connective tissues were immediately moistened with distilled water or in some cases with physiological saline. Grey translucent portions alone were selected. Care was taken to avoid unduly tough or white portions of fascia or any fat. Pieces about 5 mm wide were picked up with forceps, snipped off with scissors and immediately placed in 10 ml. quantities of the fluid in which they were to be examined. Microscopic preparations were made after not less than half an hour's immersion at room temperature. Two types of manipulation were used: either the tissue was teased and shredded on the slide before mounting or it was merely spread out by gentle pressure on the coverslip. Air bubbles were always avoided. The edges of the preparations were sealed with hot paraffin wax. The preparations were examined with a one twelfth inch objective having an iris diaphragm stop and a Leitz "cardioid" type of dark ground condenser. A pointolite was used as illuminant.

MORPHOLOGICAL OBSERVATIONS

Appearances in distilled water at pH 7·0. These are illustrated in figs. 3 and 4. It may be seen that the main mass of the tissue is composed of interwoven bundles 2–10 μ in diameter. The bundles are evenly illuminated and compact. Between is a network of fibrils,

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many of which are in continuity with the fibrils composing the bundles. The spaces between the bundles appear also to be filled with a faint cloudy opacity, best seen at the edge of the preparation (fig. 4). Elastic fibres where present are easily recognised by their straight course and angular branching. Fibrocytes and wandering cells are inconspicuous and most of them are distorted by osmotic swelling. Macroscopically the connective tissue in distilled water is grey and translucent; it spreads and teases easily and is moderately retractile.

Appearances in increasing concentrations of hydrogen ions. On account of the antagonistic action of salts to that of H ions (described below) the use of a buffer solution was impracticable. Dilute concentrations of hydrochloric acid were therefore made to approximate *p*H values, the actual *p*H being measured after the experiment either colorimetrically or with a glass-electrode apparatus. Between *p*H 7 and 5 the changes are not marked, the collagen bundles become slightly more condensed and the refractile elements in them, instead of being evenly distributed throughout the bundles, tend to become concentrated at their edges. The bundles also show slightly increased waviness. Macroscopically no great alterations are noted.

Appearances in concentrations around N/10,000 HCl. At this concentration, beginning quite abruptly, in so far as can be judged, at *p*H 4·2, the tissues are grossly and remarkably altered. Macroscopically the pieces lose their grey translucency and become opaque, almost as if coagulated (*cf.* figs. 1 and 2). Such pieces are difficult to tease apart, the constituent fibres tending to cling together as though coated by a white gummy substance. Examined with the dark-ground microscope, the edge of such a preparation appears as shown in fig. 5. The bundles and fibres are obscured by a general white opacity. Where torn apart by teasing the appearance is that of fig. 6. The bundles, some of them increasingly wavy, appear contracted and covered with a white opaque substance. A similar change is seen to affect the interfascicular fibrils. There is no trace of acid swelling: on the contrary, the general picture is that of narrowing and condensation, with greatly increased optical opacity.

Appearances at pH 4·0-3·6. In hydrogen ion concentrations more acid than 3·8 the tissues again become increasingly grey and translucent. The reason for this may be seen microscopically in the fact that acid swelling of the bundles and the fibrils has begun. Over this *p*H range there is clear evidence of the operation of the two contrary processes, the increased swelling of the collagen fibres being partially counteracted by a process of shrinkage. The appearances are well illustrated by fig. 7.

Appearances at pH 3·5-3·0. Within this range of hydrogen ion concentration, acid swelling completely overshadows shrinkage; remains of the white opaque substance may be seen in granular form in the swollen bundles and as constricting rings and cross garters within and around them (figs. 8 and 9). Almost identical illustrations may

FIG. 1.—Naked eye appearance of a piece of rat connective tissue in normal saline.

FIG. 2.—The same piece of tissue after 10 mins. in $N/10{,}000$ HCl. The opacity is due to condensation of the cementing substance. These changes produced by the antagonistic action of salts and hydrogen ions on the cementing substance are reversible.

FIG. 3.—Rat connective tissue in distilled water at pH 7·0 : middle of the preparation. It consists mainly of fibrils less than 1μ thick, partly free and partly combined in compact bundles 2-10 μ thick.   ✕ 500.

FIG. 4.—Rat connective tissue in distilled water at pH 7·0 : edge of preparation. ✕ 500.

FIG. 5.—Rat connective tissue at pH 4·0 ($N/10{,}000$ HCl) : edge of preparation to compare with fig. 4.   The fibrils and fibre bundles are obscured by a white opacity due to condensation of the cementing substance around them.   ✕ 500.

FIG. 6.—Rat connective tissue at pH 4·0 ($N/10{,}000$ HCl) : middle of preparation to compare with fig. 3.   The fibres have been teased out free from the opaque cementing substance.   The bundles are very compact and crimped owing to contraction of the cementing substance within them.   ✕ 500.

INTERSTITIAL CEMENTING SUBSTANCE

Fig 1.

Fig. 2.

Fig. 3

Fig 4

Fig. 5.

Fig. 6.

be seen accompanying Flemming's paper. Macroscopically the tissues are grey and translucent, rather resembling globules of mucus.

Salt effects. Tissue treated with physiological saline at pH 7·0 becomes grey, more translucent, and almost mucinous in consistency. Microscopically the appearance is as shown in fig. 10. The cells show up well and retain their normal contours. The bundles are notably more open textured and their constituent fibrils may be more easily identified. The fibrils give a general appearance of being more loosely woven. With a slight increase in salt concentration the loosening of texture becomes more apparent. At concentrations greater than $M/8$ the fibrils constituting the bundles frequently show Brownian movement. This effect was studied using several different salts. It was found that salts containing divalent and trivalent anions are slightly more effective in producing this effect. Little difference is observed between kations, with the notable exception of lithium. It is probable that the ease with which Brownian movement of the fibrils is produced depends upon the size and concentration of the particles bombarding them. This would account at least for the relative ineffectiveness of lithium salts.

The unravelling effect at a concentration of $M/2$ NaCl upon the constituent fibrils of connective tissue bundles is shown in fig. 11.

Antagonistic effects of salts and hydrogen ions. It is apparent that salts and hydrogen ions produce quite opposite effects in their action upon the connective tissue bundles. In the pH range 7·0–3·8 the bundles become increasingly narrow, condensed and crimped, whereas in concentrations of neutral salts greater than $M/8$ the bundles become broad, straight and unravelled and their constituent fibrils can be easily identified. Up to the point at which acid swelling of the collagen fibrils occurs (about pH 3·8) these changes are found to be reversible in either direction. That is to say, bundles narrowed and crimped at pH 4·0 become broad and unravelled when transferred to a neutral solution of $M/8$ NaCl, and vice versa. Bundles which have undergone acid swelling, however, never fully recover their former appearance when treated with salt. They remain opaque and granular.

Summary of morphological observations

It must be concluded from these observations that, in addition to acid swelling of collagen, hydrogen ions produce another quite different effect on loose connective tissue. In pH concentrations insufficiently acid to produce acid swelling, the general effect is one of condensation and shrinkage both of the tissue as a whole and of its constituent bundles. Within the pH range in which acid swelling begins (<3.8) there is clear evidence of the simultaneous operation of two antagonistic processes, namely, swelling and contraction. The irregular beaded swellings of collagen bundles in acids of strengths around 0·001 N would seem to be a resultant of these two processes.

PLATE LXXXI

FIG. 7.—Rat connective tissue at pH 3·6. Two processes are in evidence : condensation of cementing substance with increased opacity, and acid swelling of the collagen with reduction in opacity. ×500.

FIG. 8.—Rat connective tissue at pH 3·0. Acid swelling of the collagen in the bundles is predominant. The condensed cementing substance remains in the form of granules within the swollen bundle and as constricting cross garters around it. ×500.

FIG. 9.—Rat connective tissue at pH 3·0. Acid swelling of collagen bundle constricted by rings of condensed cementing substance. ×500.

FIG. 10.—Rat connective tissue in normal saline. In solutions of salt the fibrils in the bundles are less compactly woven than in distilled water. ×500.

FIG. 11.—Rat connective tissue in $M/2$ concentration of NaCl. The fibrils in the bundles have become unravelled owing to thinning of the cementing substance. As the result of the reduction in viscosity of the cementing substance, fibrils in such concentrations of salt frequently show Brownian movement. ×500.

FIG. 12.—Fibrils composing a tendon in a rat's tail. The fibrils are not held together in compact bundles and are easily teased apart. It is probable that these fibrils, unlike those of loose connective tissue, are not combined with cementing substance. ×500.

INTERSTITIAL CEMENTING SUBSTANCE

FIG. 7.

FIG. 8.

FIG. 9.

FIG. 10.

FIG. 11.
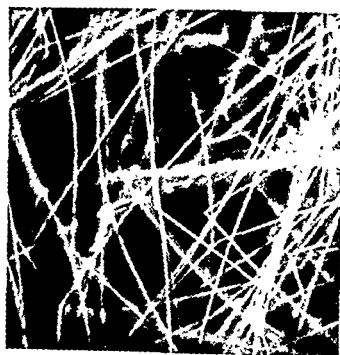
FIG. 12.

or salt linkages are considered to be comparatively strong but have the defect of being readily susceptible to ionic penetration. This accounts for the ease with which the swelling of collagen fibres occurs in acid and alkaline solutions. Discussing this subject the late Dr Dorothy Jordan Lloyd (1941) asked the question " if the fibres of muscle and the collagen fibres of connective tissue or tendons are held together mainly by salt linkages and show this strong capacity for swelling, how is it that, when dissected from the body, they form such very coherent organs with no tendency whatever to dissolve away if suspended in water or physiological salt solution ? " This question is of fundamental importance to pathology since, if collagen fibrils showed the tendency to dissolve in water which might be expected from the intrinsic chemical structure of collagen, œdema should lead to tissue disintegration. The answer which Dr Jordan Lloyd gave to this question in relation to the swelling of entire tendons of rats' tails was that the tendon is ensheathed by a substance which, as a descriptive term, she referred to as reticular tissue, this sheath being relatively resistant to acid swelling.

It has been possible to confirm the existence of such a sheath in this situation. Rat-tail tendons swollen in acid show an irregularly beaded appearance. Between the beaded swellings one can see residual pieces of the sheath which have retracted to form garters. The sheath surrounding the rat-tail tendon can be beautifully demonstrated by brief treatment of the tendon with aniline blue. Microscopical examination of such preparations shows a network of blue-stained fibres coursing over the tendon, quite different from the longitudinally arranged fibrils of the tendon itself.

There is good reason, therefore, to suppose that the collagen fibrils of tendon are protected from the disintegrating influence of hydrogen ions by a resistant wrapping which ensheathes the entire tendon. No such arrangement was found in loose connective tissue. Dark-ground examination of preparations mounted in neutral distilled water or physiological saline revealed no trace of any sort of sheath around the bundles of collagen fibrils. Constricting rings and cross garters appeared only in tissue treated with acid ; moreover, these were seen to lie not only upon but also within the bundles. There is every reason to think that Flemming was right in considering these appearances to be due to coagulation of cementing substance.

Dark-ground study also revealed significant differences between the mode of arrangement of the fibrils in tendon and in loose connective tissue. The individual fibrils of a tendon, although closely packed together, are easily teased apart. Dark-ground examination of a teased-out tendon (fig. 12) showed that the collagen fibrils are not combined into bundles but stand out clearly as individual structures. It was also seen that the fibrils of tendons swollen in acid show no constrictions or irregularities. The fibrils merely become progressively

disintegrated without the appearance of anything suggesting the coagulation of cementing substance.

It is probable, therefore, that the individual fibrils of which a tendon is composed are not combined with cementing substance but are protected from disruptive ionic influences by a sheath which surrounds the entire tendon. On the other hand the fibrils of loose connective tissue would appear to be intimately combined with cementing substance and it is to this substance that the bundles owe their cohesion in an acid or otherwise disruptive chemical environment.

A further point to be discussed is the relationship between the cementing substance and the amorphous ground substance demonstrated in a variety of ways by Bensley (1934). Nageotte (1922) has denied its existence, but his paper on this subject does not contain as emphatic a denial as the title would suggest: ("Il n'y a pas de 'substance amorphe' dans la trame conjonctive"). In this communication he states that the only components of the connective tissue visible with the microscope are networks of fibres of decreasing size. He assumes, therefore, that any histologically amorphous substance present would have the same essential fibrillary structure. This conception is in full accordance with the recent work of protein chemists. Thus Jordan Lloyd (p. 284) :—" Most of the protein fibres are sufficiently large to be seen by the naked eye, certainly by a low-power lens of a microscope. They have nearly all been split by suitable means into bundles of fine parallel fibrils. These are sometimes visible under a low-power lens, but sometimes require a high-power lens. These fibrils can be split again into bundles of parallel sub-fibrils that sometimes can only be made visible by the ultra-microscope. There is no doubt that by pursuing the splitting process far enough the parallel bundles of long thin molecules, already described, would be reached ". It is apparent that in the study of proteins the fields of histology and modern structural chemistry are converging. Even to a histologist, the term structure is coming to mean not merely micro-anatomical structure but also molecular configuration. From this point of view the question as to whether a given tissue component appears microscopically to be amorphous or fibrillary is of less importance than the question of what molecular arrangements are present and what is the function of these arrangements. There may in fact be a variety of amorphous substances in connective tissue. The work of McClean (McClean and Hale, 1941) has shown that hyaluronic acid is present in quantity in the skin, since an enzyme acting upon this substance is responsible for the rapid diffusion of substances injected intradermally. Modern conceptions of fibrillogenesis would also demand the existence of a histologically amorphous substrate, out of which the fibres form. The cementing substance described may in a sense be considered part of the histologically amorphous element in connective tissue, but it

is not the only substance which may be present in histologically amorphous form.

SUMMARY

Dark-ground microscopical studies of rat interstitial connective tissue has confirmed the existence of a cementing substance as described by Flemming 70 years ago. This substance is digested by trypsin, shows maximal condensation at pH values between 4·2 and 3·8, and swells, with reduction in viscosity, in concentrations of neutral salts of $M/8$ and over.

REFERENCES

ASTBURY, W. T.   .   .   .   .   .   1940.   Molecular structure of the fibres of the collagen group, *London*, p. 9.

BENSLEY, SYLVIA H.   .   .   .   .   1934.   *Anat. Rec.*, lx, 93.

FLEMMING, W.   .   .   .   .   .   .   1876.   *Arch. mikr. Anat.*, xii, 434.

LLOYD, DOROTHY JORDAN   .   .   1941.   *J. Soc. Dyers and Colourists*, lvii, 281.

McCLEAN, D., AND HALE, C. W.   .   1941.   *Biochem. J.*, xxxv, 159.

NAGEOTTE, J.   .   .   .   .   .   .   1922.   *Compt. rend. Soc. de biol.*, lxxxvii, 147.

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AGE CHANGES IN LYMPH NODES

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(PLATES LXXXII-LXXXVI)

THAT profound changes occur in lymphoid tissue in ageing has long been appreciated, and the literature up to 1930 has been reviewed by Hellman. Detailed accounts of the ageing of the spleen, appendix and tonsils are available but reports on the lymph nodes are fragmentary, ill-co-ordinated and often contradictory. In this paper the histological changes in the lymph nodes are traced from infancy to senility.

MATERIAL FOR EXAMINATION

This comprised over 300 lymph nodes collected from 150 autopsies, augmented by a small number of normal glands obtained by biopsy. About one-third of these nodes came from accident cases, one-third from acute medical and surgical cases, and one-third from subjects of chronic disease. The use of such material has been condemned by Hellman (1930) as unlikely to lead to reliable conclusions on normal structure, but any survey of this nature should include nodes from both the healthy and the sick, and should comprise the greatest possible number consistent with their adequate study. Over 200 of the glands belonged to the deep cervical and inguinal groups. The remainder were bronchial, mesenteric or axillary.

METHODS

The material was fixed in formol-saline, Bouin's fluid or Helly's fluid before paraffin embedding. Staining methods included Ehrlich's acid hæmatoxylin and eosin, van Gieson, Mallory's phloxin methylene blue and Laidlaw's reticulum method. Over fifty nodes were studied in serial sections. In some cases large scale models in plasticine were made from serial drawings, the outlines of these drawings being pricked through the paper on to thin sheets of plasticine of known thickness. The design was then cut out, the pieces of plasticine superimposed on a squared pattern and the model built up to an appropriate thickness. The model pictured in fig. 1 was built up in this way.

THE NORMAL LYMPH NODE

A precise knowledge of the individual tissues comprising the lymph node is essential for an understanding of age changes. In this investigation the relations of the lymph sinuses, lymphoid tissue, blood vessels and fibrous and reticular tissues have been studied.

Lymph sinuses

The afferent lymphatics pierce the fibrous capsule of the gland and enter the marginal sinus which completely surrounds the lymphoid tissue. This marginal sinus is a lymph-containing space about 40 μ in width, continuous with the hilar lymphatics and in the foetal gland traversed only by reticulum fibres. The trabeculæ which appear at the age of six months are fibrous-tissue pillars arising from the capsule and passing in the lumina of wide intra-glandular lymph sinuses to the hilum. The continuity of the marginal sinus is further disturbed at a much later age in the superficial nodes by the approximation of the lymphoid tissue to the capsule in many areas, so that the marginal sinus then forms a series of intercommunicating loculi.

The mass of lymphoid tissue is traversed by intra-glandular lymph sinuses which pass from the marginal sinus to the hilar lymphatics. These lymph sinuses form a series of tortuous anastomosing channels 40-70 μ in diameter. In sections of the adolescent gland, where the lymphoid tissue is well developed, sinuses occupy a very small part of the gland, which appears as a continuous sheet of lymphoid tissue condensed here and there to form germinal centres and interrupted by portions of lymph sinuses caught in transverse section. A two-dimensional view of lymph sinuses gives an inadequate picture of their number and complexity, a complexity that is displayed in fig. 1. This system of sinuses can be divided into three parts—cortical, medullary and hilar.

Cortical sinuses. The intra-glandular lymph sinuses pass radially from the marginal sinus to the medulla, but even in the cortex anastomosis is frequent and in travelling a distance of less than one mm. each sinus becomes joined to four or five others.

Medullary sinuses. In the medulla, the sinuses anastomose more freely so that all sense of orderly arrangement is lost. The lymphoid tissue is reduced to narrow medullary cords and the proportion of sinus to lymphoid tissue is much greater than in the cortex.

Hilar sinuses. The hilar structure varies considerably in different groups of glands and at different ages. The hilum consists of efferent lymphatics and veins, afferent arterioles and a variable amount of connective tissue. This structure usually intrudes some distance into the gland. The marginal sinus is infolded at the hilum and insulates the hilar intrusion from the lymphoid tissue. In the depths of the gland, where the hilum breaks up, the marginal sinus joins the terminations of the intra-medullary sinuses to form the efferent lymphatic vessels. The hilar fibrous tissue is always separated from the lymphoid tissue by lymph sinuses.

The simplest form of hilar structure is seen in the inguinal lymph nodes (fig. 2). The lymphoid tissue of these glands has a cup-like arrangement and is surrounded both externally and internally by lymph sinus. The hilar structures which fill the concavity of the

AGE CHANGES IN LYMPH NODES
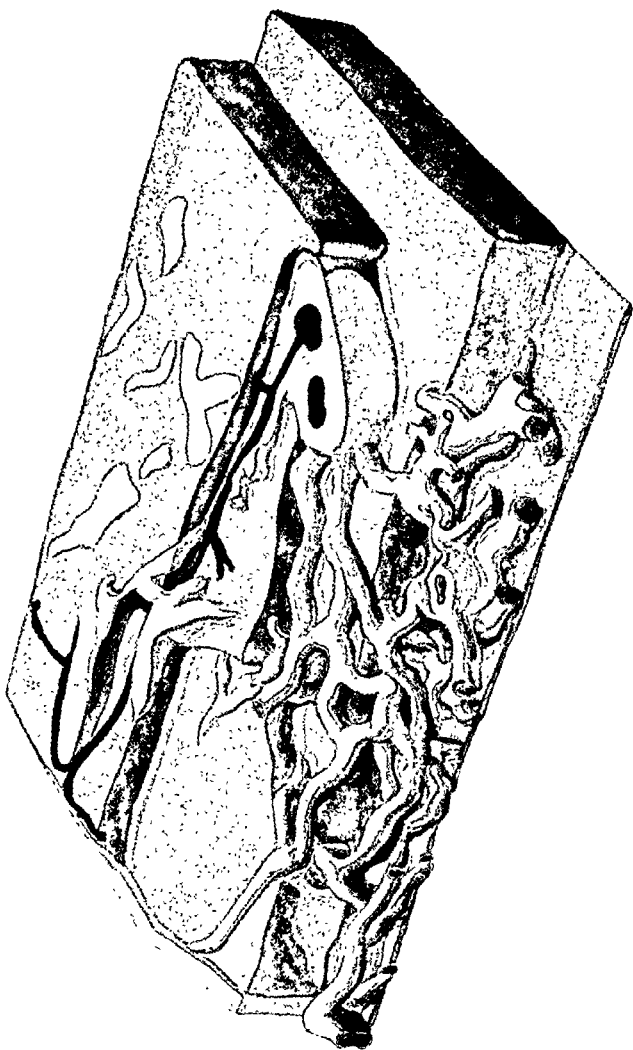
FIG. 1.—Drawing of model of a lymph node to show the arrangement of the lymph sinuses and the continuity of the lymphoid tissue. The marginal sinus is shown to the left and on the right a hilar protrusion contains an artery and a vein. ×50.

cup are thus insulated from lymphoid tissue. The concave shape and
the nature of the blood supply limit the thickness of lymphoid tissue.
The large fleshy glands of the deep cervical group have a different
hilar structure, providing adequate nutrition for the great aggregation
of lymphoid tissue. The main hilum is superficial, but small hilar
protrusions reach far into the glandular tissue (fig. 3). These form
the medullary trabeculæ and carry the afferent and efferent blood
vessels of the lymphoid tissue. The medullary trabeculæ are branching
structures many mm. in length which become continuous with the
cortical trabeculæ. Each hilar protrusion consists of a fibrous-tissue
core surrounded by a lymph sinus. Within the fibrous tissue lie an
arteriole and a vein, which part company as the accessory hilum
divides and redivides, and then run in separate trabeculæ (Hendorfer,
1921). Along the course of the arterioles branches are given off to
cross the lymph sinus and enter the lymphoid tissue. In this way
blood is carried into the depths of the gland. By the multiplication
of accessory hila, a gland may increase to a diameter of several cm.
while any portion of lymphoid tissue will still be within one mm.
of an arteriole running in an accessory hilum. Some large glands
possess more than one hilum and appear to develop by the fusion
of several small units as described by Teichmann (1861), Kling (1904)
and Hammerschlag (1908).

Lymphoid tissue

The collection of cells forming the lymphoid tissue is supported
by a reticular meshwork and is cushioned from the capsule by the
marginal lymph sinus. As shown by Bunting (1904-05) the mass of
lymphoid tissue is continuous, being pierced but not interrupted by
lymph sinuses. In sections, areas of lymphoid tissue are often caught
fortuitously between two radially arranged lymph sinuses. Such
areas are described as primary follicles, but this definition is deceptive,
suggesting as it does that this block of lymphoid tissue is walled off
by the sinuses. If a section is taken deeper in the block these sinuses
will have disappeared to be replaced by other sinuses in new positions.
The essential continuity of lymphoid tissue, both cortical and
medullary, is shown in fig. 1.

The distinction between cortex and medulla is usually made by
reference to the difference in arrangement of the cortical and medullary
sinuses. In ordinary hæmatoxylin-stained sections it is often difficult
to distinguish between the two zones. Silver staining discloses obvious
differences between the cortical and medullary reticulum (fig. 4).
Distinction between the cortex and medulla is relatively easy in the
superficial glands, where the cortex uniformly surrounds the medulla ;
it is more difficult in the deeper glands, where the cortex reaches, in
an irregular manner, far into the substance of the glands.

Reticulum

In accordance with modern convention, the term reticulum is used to describe the argyrophil fibres rendered black by ammoniacal silver solution. The reticular arrangement at birth is described, for the changes in ageing are imposed on this fundamental structure. The reticulum can be divided into that of the marginal sinus, the cortex and the medulla.

Marginal sinus. Reticulum fibres are the only structures traversing this sinus at birth. They arise from the fibrous capsule, cross the marginal sinus in irregular radial fashion, and just before joining the cortical reticulum divide into two or three fibres that gain independent insertion in the reticular network of the cortex. The lymphoid tissue of the gland is suspended in a bath of lymph by these fibres, about 10,000 of which cross each sq. mm. of the marginal sinus to support the lymphoid tissue.

Cortical reticulum. The cortical reticulum is arranged as an open meshwork of fibres (fig. 5). The average diameter of this meshwork is 20 μ (compare values of 12-20 μ by von Ebner (1902) and 10-30 μ by Jolly (1923)). Orsós (1926) has stated that these fibres are arranged to enclose rhombic dodecahedra. But Kelvin (1904) has shown that other polyhedra possess even greater economy of surface to volume. D'Arcy Thompson (1942) has demonstrated in invertebrates that although one polygonal form may predominate in the fibrous structure, other polyhedra are also present. The arrangement of the reticular fibres in the lymph nodes provides the most economical use of fibres to support the lymphocytes and other cells of the tissue, but this arrangement is probably less regular than Orsós has suggested.

Medullary reticulum. The medullary reticulum is characterised by a much closer meshwork than the cortical reticulum (Richter, 1902; Balabio, 1908; Rössle and Yoshida, 1909; Orsós, 1926). This mesh is slit-like, with an average diameter of 6-8 μ, so that the medullary reticulum has a much darker appearance under the low power of the microscope (fig. 6). This difference in cortical and medullary reticulum disclosed by silver staining enables a clear distinction between cortex and medulla to be made' and changes in their relative proportions to be detected.

Both medullary and cortical reticulum are interrupted by two structures—blood vessels and intra-glandular lymph sinuses. The former are almost exclusively capillaries and venules. Each vessel has an intimate investment of reticulum, the thicker fibres of which are wound spirally around the vessel. But there are many lateral connections, so that the reticular arrangement is net-like. As a result of this close reticular investment, the vessels give the appearance in silver-stained sections of cylinders of reticulum. From these cylinders thick fibres are given off to the general cortical and medullary

AGE CHANGES IN LYMPH NODES

Fig. 2.—Inguinal node at age 24, showing a central fibrous hilum covered by an inner darker layer of medullary reticulum and an outer lighter layer of cortical reticulum. Laidlaw. ×8.

Fig. 3.—Deep cervical node at age 20, showing trabeculae and intermingling of light cortical and dark medullary reticulum. Laidlaw. ×12.

Fig. 4.—Fœtal node showing the clear distinction between the light cortical reticulum and the dark medullary reticulum. Laidlaw. ×40.

reticulum, and in this way the vessels are fixed to the reticular framework of the node.

Intra-glandular lymph sinuses are canals lined by reticulum that in cross section appears to be arranged as longitudinal fibres, but in sagittal section is seen to form a close net with slit-like mesh. In fœtal glands very few reticulum fibres traverse the intra-glandular lymph sinuses. Later, transversely arranged fibres appear but are so irregular in arrangement and distribution that the relative numbers of fibres at different sites cannot be measured accurately (Toldt, 1888 ; Thomé, 1902). But Bartel and Stein (1905) have stated that the reticular mesh within the medullary sinuses is finer than within the marginal sinus. This is certainly not the case at birth and is probably incorrect for glands of any age, but variations in individual glands are considerable and so many other factors enter into the arrangements of the sinuses and their reticulum that the value of such measurements is very doubtful.

Connective tissue

The fibrous capsule is directly continuous with the hilar connective tissue. In the fœtal gland it is separated from the lymphoid tissue by the marginal sinuses, but after puberty this separation may become incomplete. The blood supply of the capsule is derived from the hilum directly, and from the surrounding connective tissue. In the young node the capsule does not receive any blood supply via the lymphoid tissue, but after puberty this clear isolation of capsule from lymphoid tissue may be lost and small vessels then cross from lymphoid tissue to capsule where these are in contact.

Trabeculæ are rarely evident in the fœtal gland and *do not pass completely from capsule to hilum*. As the gland increases in size hilar trabeculæ grow into the depths of the gland and *by 6 months of age* have become continuous with the cortical trabeculæ arising from the capsule. In the inguinal nodes the trabeculæ are poorly developed : in the deep cervical they form a fibrous network, but only *a small proportion* of the intra-glandular lymph sinuses contain them.

Blood vessels

The artery supplying the node enters at the hilum and divides into arterioles that run in the hilar trabeculæ deep into the gland. At intervals, branches in the form of pre-capillary arterioles are given off to traverse the investing lymph sinus and to enter the medullary portion of the lymphoid mass. Once in the lymphoid tissue the course of the pre-capillary arteriole is characteristically straight, passing from medulla to cortex, a distance rarely exceeding 2 mm. Along its course it gives off many capillaries and terminates as part of the capillary network of the cortex. The capillary network in the lymphoid tissue consists of a closely anastomosing series of vessels.

The venules draining this plexus are more tortuous in their path than the pre-capillary arterioles, and join the veins lying within the trabeculæ. In most cases the veins do not occupy the same trabeculæ as the arterioles (Heudorfer) except near the hilum.

Germinal centres

The presence of germinal centres exerts such a profound influence on the structure of lymph nodes in relation to their age that some account of their characteristics is essential if the age changes in lymph nodes are to be understood. Classifications for the various types of germinal centres have been proposed by Groll and Krampf (1920-21) Rotter (1927), Ehrich (1929) and Fischer (1937). In this paper the simple subdivision by Maximow (1932) into active centres of the Flemming (1885) type and resting centres will be used. The controversies concerning nomenclature and function of germinal centres will not be considered, but inherent in the discussions and conclusions in this paper will be the conviction that the active centre with its pale core and compressed periphery of reticulum and darkly staining lymphocytes bears the mark of active proliferation and the resting centre the signs of exhaustion of this activity.

In this series the germinal centres made their appearance at an age of 5-6 months, as compared with the range of 2-6 months given by Gundobin (1906), Barnes (1909), Nagoya (1913), Hellman (1921), Foerster (1923), Wetzel (1926) and Ehrich (1929). The centres reach their maximum development in childhood between 5 and 10 years, persist in the active stage until adolescence and then appear in the resting phase, reduced in size and number, but persisting in most lymph nodes for the rest of the life of the individual. This persistence, despite nutritional and other disturbances, has been observed by Firleiewitsch (1906), Gaetano (1928) and Berggren and Hellman (1930).

Although the various forms of germinal centre have been defined in detail, no clear account has been given of the process of their development and duration of life. Labbé (1898) and Ehrich (1929) state that the centres arise as small cortical condensations of lymphocytes in the centre of which appear large pale-staining cells whose subsequent proliferation produces the active centre. Retrogression is thought to occur as a result of reduction in central mitosis, a steady decrease in size and gradual replacement of the large pale cells by small lymphocytes, to give the picture of a small dense aggregation of lymphocytes at first standing out in relief but finally disappearing by diffuse dissolution into the surrounding lymphoid tissue (Levinstein, 1909; Renn, 1912). Maximow (1932) has suggested that cyclic changes occur in germinal centres, the centre passing from resting to active phase and back again, but he does not discuss the time occupied by these changes. Conway (1937) has shown in experimental animals that this cyclic process may be speeded up to be complete in a few days.

AGE CHANGES IN LYMPH NODES
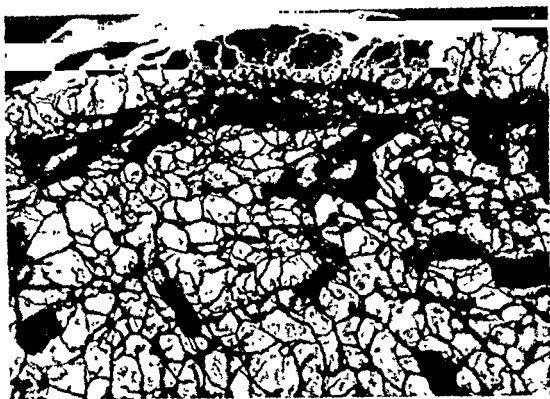
FIG. 5.—Fœtal node showing marginal sinus and open meshwork of cortical reticulum. Laidlaw. ×250.

FIG. 6.—Fœtal node showing transition between open cortical reticular mesh (above) and fine medullary mesh (below). Laidlaw. ×250.

FIG. 7.—Inguinal node at age 58. Normal subject. Hilar tissue entirely fibrous. Laidlaw. ×10.

Flemming implied that germinal centres developed in fixed sites in lymph nodes, and Maximow (1927) stated that this localisation was the result of vascular distribution, each germinal centre developing around a special blood vessel. The experiments on the active immunisation of animals by Hellman and White (1930) support this conclusion and suggest that the germinal centres are fixed in number as well as position. They demonstrate that the number of germinal centres is no greater in inoculated than in control animals, but the centres are much larger in the former group. Conway stated that germinal centres are neither fixed in location nor limited in number, and that no constant relationship with blood vessels could be found. A number of investigators (Hueck, 1927, 1928 ; Röhlich, 1928 ; Jäger, 1928-29 ; Schmelzer, 1936) have studied the blood supply to germinal centres and conclude that there is always an associated afferent arteriole. But it should be emphasised that germinal centres may be very large structures, up to 1 mm. or more in diameter and, as a result of the space they occupy, they are almost certain to be in contact at some part of their circumference with one or more of the pre-capillary arterioles which are so uniformly arranged in lymph nodes.

Germinal centres are a specialised part of lymphoid tissue— according to Foerster the last and highest product of differentiation of that tissue. The cortical reticulum appears to have a limit to its degree of distension with cells, and cellular proliferation beyond this limit results in local disruption about the focus of cellular proliferation which forms the germinal centre. Further disproportional growth of the centre leads to peripheral compression of the reticulum. That germinal centres are not confined to the cortex but may be found in the medulla has been frequently reported (Labbé, 1898 ; Bunting, 1904-05, Heudorfer, 1921 ; Oeller, 1928 ; and many others). The arbitrary distinction between cortex and medulla is difficult to maintain and the inter-relation of cortex and medulla is often complex, but it seems possible that the medullary reticulum may be transformed to the cortical type, behaving as a collapsed lattice that on suitable stimulation may be re-opened to form the fully distended cortical form of reticulum. Further local distensions will result in areas of disruption to form germinal centres. Even when the medullary reticulum has undergone some collagenous change, cellular proliferation may distend it to capacity and even to disruption, so that between the germinal centres and amid the proliferated lymphocytes fragments of collagenous fibres 'may be seen, an indication of the relatively acellular structure of the gland before the stimulus to growth provoked the cellular reaction.

The germinal centres are distributed in lymph nodes either at the periphery of the gland in close association with the marginal sinus, or surrounding as a sleeve the large trabeculated intra-glandular lymph sinuses. In this way the germinal centres are apparently related to the distribution of afferent lymph within the lymph node (Chievitz,

changes occurring in axillary nodes during lactation (Stiles, 1892-93; Gulland, 1893-94, 1894).

The lymph nodes can be separated into the two main groups, superficial and deep. These show essential differences in structure and in response to ageing. The inguinal glands are here taken as representative of the superficial group and the deep cervical glands of the deeper group, and the age changes are described independently. From the material examined in the present investigation it can be concluded that the maximum size of lymph nodes is reached in childhood at an age of 7-10 years, supporting the conclusions of Hellman (1930), who on the basis of experimental work with animals and a review of the literature on human nodes decided that the greatest development was reached before puberty. In the investigation of the anatomical changes in ageing, the differences in the regional groups of nodes must be considered.

AGE CHANGES IN INGUINAL NODES

The inguinal nodes have a cup-like shape and a fibrous hilum that projects into the gland to fill the cup. This characteristic structure is often evident at birth and can always be seen at the end of six months, as by this time a few trabeculæ may have developed, but inguinal nodes are poorly trabeculated. At six months the lymphoid tissue has increased sufficiently to contain a few small germinal centres in the cortex. Until adolescence there is a steady increase in lymphoid tissue until the gland reaches a length of 1 cm. or more. The hilum passes into the gland for about 0·5 cm. The rind of lymphoid tissue attains a thickness of several mm., and where this is exceeded, medullary trabeculæ appear. Germinal centres become more numerous but they are always less well developed than in the deep nodes. The germinal centres of the inguinal nodes are fewer in number, smaller in size, show less reticular compression and possess smaller pale centres than those of the deep cervical nodes. The germinal centres appear in the cortex which, at this period, is wide and continuous (fig. 2). As the cortex tends to follow the hilum into the gland, germinal centres may be distributed along the line of the hilum as well as around the periphery of the node. Exceptionally, centres may be found deep in the gland.

Retrogression commences at puberty. The lymphoid tissue decreases steadily in amount, but although the total mass of this tissue may diminish to a tenth or even less, there is no great reduction in the over-all size of the gland, due to the fact that in retrogression the lymphoid tissue shrinks on to the capsule rather than on to the hilum. In this way a narrowing rind of lymphoid tissue lines the capsule and the cup is filled with increasing amounts of connective tissue. This method of shrinking is a fundamental property of matter. D'Arcy Thompson (1942, p. 562) has shown that spherical

AGE CHANGES IN LYMPH NODES

FIG. 8.—Inguinal node at age 62. Fat subject. Replacement of hilar fibrous tissue by adipose tissue. Laidlaw. × 10.

FIG. 9.—Inguinal node at age 71. Wasted subject. Enlargement of lymph spaces of medulla and hilum. Laidlaw. × 10.

FIG. 10.—Inguinal node at age 33. Tangential section to show islands of open cortical reticulum in denser medullary reticulum. Laidlaw. × 50.

objects shrink to form perfect cup-like figures and has used this fact to explain the formation of the gastrula stage of the embryo.

The nature of the hilar tissue filling the increasing cavity of the lymph node varies with the nutritional state of the individual. In the normal individual the hilum is composed almost entirely of collagenous connective tissue (fig. 7). Where there is much fat stored in the body the hilar fibrous tissue is replaced by adipose tissue (fig. 8). A series of gradations between a completely fatty and a completely fibrous hilum is found and is closely related to the degree of general adiposity. Several writers, reviewed by Hellman (1930), have discussed the accumulation of fat in lymph nodes and some have considered that the fat may actively displace the lymphoid tissue, but Sternberg (1926) has pointed out that fatty accumulation may occur in the absence of reduction of lymphoid tissue. It seems much more likely that the body fills the lymphoid cup with the connective tissue available—the spare individual with fibrous, the obese with adipose tissue. In starvation or wasting diseases, where the nutritional state of the body is poor, the hilum becomes cystic (fig. 9). The connective tissue is greatly reduced, and in its place great lymph-containing spaces are found, but the lymphoid tissue is still peripheral in arrangement.

The diminution of lymphoid tissue results from a decrease in the cellularity of the gland (Rössle and Yoshida) with a relative increase in the fibrous element (Jolly). This is illustrated by the relatively greater reduction in cortex than in medulla. The cortex with its open meshwork has always a more cellular structure than the closer-meshed medulla. After the age of 20, germinal centres have almost entirely disappeared from inguinal nodes, although a few inactive centres may persist, but the thickened reticular fibres that arose from the capsule and joined the peripheral condensation of reticulum about the germinal centres persist. These fibres occur in bundles and usually pass through the cortex to join the medullary reticulum. Shrinking of the cortex proceeds, so that at the age of 40 it ceases to be continuous and in places the medulla, reinforced by the trajectorial fibres, reaches the marginal sinus. Here capillaries cross with the trajectorial fibres to the capsule, thus breaking down the vascular isolation of the lymphoid tissue from the capsule. Lymphocytes appear in the meshwork of these reticular pillars and in places the lymphoid tissue comes into actual contact with the capsule. The intracapsular proliferation of lymphocytes becomes possible by direct continuity with the lymphoid tissue when the gland is stimulated to new activity. The marginal sinus is no longer continuous, but is now interrupted by pillars of lymphoid tissue showing the reticular arrangement and reduced cellularity of medullary tissue.

The reduction in cortical tissue continues at a slower rate after forty. At first the cortex is continuous, but the medullary intrusions show a relative increase in size until the cortical tissue is broken up into

islands which finally have a diameter of 0·5 to 1 mm. (fig. 10). Superficially they resemble germinal centres, and indeed the islands of characteristic cortical reticulum often have a central reticular deficiency, indicating the persistence of a resting germinal centre.

Coincident with the decrease in lymphoid tissue a collagenous change occurs in the medullary reticulum and can be detected either by silver staining or by van Gieson's stain. This change commences at 30, progresses slowly and is incomplete, so that reticulum fibres are always intermingled with collagen fibres. Fibrous change does not occur until the medullary lymphocytes are greatly reduced in number and the reticulum fibres closely compacted, a condition greatly favouring collagenous change (Mallory and Parker, 1927). Age is, however, a factor, since the compacting of the reticulum occurs many years before the collagenous change commences.

The medullary fibrous tissue is separated by a lymph sinus from the hilar fibrous tissue, often conspicuously so in old age, but the complete separation of medulla and hilum that is found in childhood is lost to some degree in ageing, as is the complete isolation of cortex from capsule. In places the medullary tissues become continuous with the hilar tissues. The fibrous transformation of the medulla does not in any way alter the proliferative potentialities of this part of the node, for under the stimulus of inflammation, the fibrous medulla fills with lymphocytes and scattered, disrupted collagen fibres are seen to be overwhelmed by the newly formed lymphocytes. The collagenised reticulum of the medulla occasionally shows minimal adiposity.

AGE CHANGES IN CERVICAL NODES

The deep cervical lymph nodes differ considerably from the superficial nodes. They are spherical or spheroidal and reach a large size. The arrangement of the trabeculæ and the blood supply have been discussed above. The node is fully trabeculated at six months, when the gland is about 5 mm. in diameter and contains a few germinal centres. The node increases in size to reach its maximum at 7-10 years, when it may be 1 cm. or more in diameter. The increase in size is accompanied by further development of trabeculæ, which preserves the essential relation between trabeculæ carrying blood vessels and the lymphoid tissue. There is no hilar protrusion of lymphoid tissue as in the inguinal nodes and the relation between cortex and medulla is much more difficult to define, as has been emphasised by Nordmann for aortic nodes and Kopsch for mesenteric nodes.

The key to the cortical and medullary arrangement may be taken as the trabeculæ. In the deep cervical lymph nodes, each trabecula, contained in its lymph sinus, has a coat of cortical tissue up to 1 mm. or more thick. As the trabecula passes deeply into the medulla it carries this cylinder of cortex with it, so that the medulla

AGE CHANGES IN LYMPH NODES

FIG. 11.—Cervical node at age 78, showing similarity to fœtal node (compare fig. 4).
Laidlaw. ×25.

FIG. 12.—Cervical node at age 78; cortical reticulum unchanged throughout life
(compare fig. 5). Laidlaw. ×250.

becomes broken up by strands of cortex which anastomose as they follow the branching trabeculæ. While this process of cortical infiltration of the medulla is taking place, germinal centres are developing, for these appear at about the same time as the trabeculæ. The germinal centres arise only in the cortical reticulum, which is now not only peripheral in arrangement but also radial along the trabeculæ. Thus each trabecula, like the branch of a tree, bears the fruit of germinal centres. In fig. 3 a cross section shows considerable intermingling of medulla and cortex, but reconstruction shows that the buried cortical reticulum is always adjacent to trabecula-bearing lymph sinuses. This complex arrangement of cortex and medulla with consequent appearance of germinal centres in cortical reticulum located deeply in the gland demonstrates that most of the controversy about the presence of germinal centres in the medulla of lymph nodes is based on an inadequate knowledge of the normal structure. The clear differentiation between cortex and medulla, as here described, can be made only with silver-stained preparations where the characteristics of cortical and medullary reticulum are easily seen.

It should be emphasised that the lymph sinuses can be divided into two groups, those not bearing trabeculæ and having a diameter of about 40 μ, and trabeculæ-bearing sinuses of over twice this diameter. The smaller sinuses pass through the cortical tissue to form a complex network of sinuses which drain into the larger sinuses. The large trabeculæ-containing sinuses pass much more directly from marginal sinus to hilum. There are alternative paths for lymph. It may pass more or less directly through the gland in the large sinuses or it may take the long and tortuous path through the smaller lymph sinuses of cortex and medulla. In many lymph nodes, during the proliferative stage, the lymph sinuses within cortex and medulla are full of lymphocytes, while the broad trabeculated sinuses are free from cells. If such a gland is stained with hæmatoxylin and eosin the lymphoid tissue appears as a continuous sheet, and the intraglandular sinuses cannot be detected owing to the absence of their one distinguishing feature, emptiness. In the gland stained for reticulum the intraglandular sinuses are seen to be present in normal numbers and distribution.

In childhood, well developed germinal centres cluster around the periphery and around the trabeculæ passing into the substance of the node. At this stage the active centres described by Flemming are best seen. The nodes persist in this state of maximum development until puberty, but even at this time the germinal centres have decreased somewhat in size, with consequent decrease in size of the lymph node. From puberty onwards there is a diminution in size of the germinal centres. These do not disappear but are present as inactive forms and can still be found at the age of 80. The deep cervical nodes do not show any of the marked changes in size and structure that are seen in the superficial nodes. The statement of

Krumbhaar (1942) that most nodes go on at least to 60 years with little atrophy certainly applies to the deep cervical nodes, and probably also to other groups of deeply situated glands.

The retrogressive changes that do occur consist of a decrease in the activity and prominence of germinal centres, an increase in size of fibrous trabeculæ, and a general decrease in all elements of the gland, cellular, reticular and fibrous. In extreme old age the node sometimes corresponds in its simplicity to the fœtal gland, and cortex and medulla may again be distinguished as continuous units, and trabeculæ are almost absent (fig. 11). It is incorrect to consider a lymph node as a structure built at puberty to last until death. It is probably undergoing constant slow change, so that an infinite number of minute changes over many years produces an entirely new structure. None of the units of the node last the lifetime of the individual and there is unlimited opportunity for reconstruction.

The return in old age to the fœtal type of gland is not confined to the gross structures of medulla and cortex. The finer structure is also reproduced. Cortical and medullary reticulum have the same arrangement in the senile as in the fœtal node, and this reticulum is pierced in the same way by blood vessels and lymph sinuses (fig. 12). The medullary reticulum may show some fibrous tissue change but the open meshwork of the cortex is without collagenous taint.

SUMMARY

1. From a study of the structure of lymph nodes it is concluded that in superficial nodes, e.g. inguinal, the lymphoid tissue consists of a thin layer covering a large fibrous hilum. In deeper nodes, e.g. deep cervical, there is a greater mass of lymphoid tissue into which the hilum does not intrude but is replaced by many medullary trabeculæ which ramify within the node and carry the blood vessels into the lymphoid tissue.

2. The cortical reticulum is arranged as an open meshwork of fibres, while the medullary reticulum is a condensed system with a slit-like mesh. This difference in reticular arrangement can be used to demonstrate variations in arrangement and in relative proportions of cortex and medulla.

3. Germinal centres occur in the active form in childhood and commence to retrogress at puberty. Resting centres can be found in most glands at any age after puberty. If cyclic changes occur in germinal centres in the absence of inflammation, these changes must be slow and unimportant. Germinal centres are loci of disproportionate growth within the cortex.

4. Lymph nodes show differences in age changes dependent upon their function and their anatomical position. Superficial nodes form one group, and deep nodes which are regional to mucous membranes

another. Both groups reach their maximum development in childhood and decrease in size after puberty.

5. In inguinal nodes, trabeculæ and germinal centres are poorly developed at all ages. After puberty, the cortical tissue decreases steadily, and in old age the cortical tissue is confined to islands surrounded by medullary tissue. Despite considerable shrinking of lymphoid tissue the inguinal nodes do not show very great diminution in size, the lymphoid tissue defect being compensated by hilar connective tissue.

6. Deep cervical nodes in childhood show well developed trabeculæ and many large active germinal centres. At puberty there is some diminution in size of the nodes resulting from retrogressive changes in the germinal centres, but subsequent changes are slight and the deep cervical nodes do not show the same degree of transformation as is found in the inguinal nodes. In extreme old age there is a gradual return to the fœtal type.

7. At any period the stimulus of local inflammation may cause the node to proliferate and take on the characters of the node found in childhood.

REFERENCES

BALABIO, R. . . . . . . 1908. *Anat. Anz*, xxviii, 135.
BARNES, H. A . . . . . . 1909. *Trans. Amer. Laryngol. Ass.*, xxxi, 156.
BARTEL, J , AND STEIN, R. . . 1905. *Arch. Anat. u. Entwicklungsgesch.*, p. 141.
BERGGREN, S , AND HELLMAN, T. 1930. *Acta oto laryngol.*, supp. 12.
BUNTING, T L. . . . . . . 1904-05. *J. Anat. and Physiol.*, Lond , xxxix, 55.
CATANIA, V. . . . . . . 1927. *Hæmatologica*, viii, 221.
CHIEVITZ, J. H . . . . . 1881. *Arch. Anat. u. Entwicklungsgesch*, p. 347.
CONWAY, ELEANOR A. . . . . 1937. *Anat. Rec.*, lxix, 487.
DOWNEY, H., AND WEIDENREICH, F. 1912. *Arch. mikr. Anat.*, lxxx, 306.
VON EBNER, V. . . . . . 1902. *In* Kölliker's Handbuch der Gewebelehre des Menschen, 6th ed , Leipzig, vol. ii, p. 702.
EHRICH, W. . . . . . 1929. *Amer. J. Anat*, xliii, 347 and 385.
FIRLEIEWITSCH, M . . . . 1906. *Z. Biol.*, xlvii, 42.
FISCHER, H. . . . . . 1937. *Z. mikr.-anat. Forsch* , xli, 229.
FLEMMING, W. . . . . . 1885. *Arch. mikr. Anat* , xxiv, 50 and 355.
FOERSTER, A. . . . . . 1923. *Arch. path. Anat.*, ccxli, 418.
GAETANO, S. . . . . . 1928. *Hæmatologica*, ix, 397.
GOLDKUHL, E. . . . . . 1927. *Z. mikr. anat. Forsch.*, viii, 365.
GROLL, H., AND KRAMPF, F. . 1920-21. *Zbl. allg. Path.*, xxxi, 145.

GULLAND, G. L. . . . . . . 1893-94. This *Journal*, ii, 447.

,, ... 1894. *Rep. Lab. R. Coll. Phys. Edinb.*, v, l.

GUNDOBIN, N. . . . . . . 1906. *Jahrb. Kinderheilk*, lxiv, 529.

HAMMERSCHLAG, R. . . . . 1908. *Arch. path. Anat.*, cxciv, 320.

HELLMAN, T. . . . . . 1921. *Beitr. path. Anat.*, lxviii, 333.

,, ... 1930. Die Lymphknötchen und die Lymphknoten, *in* von Möllendorff's Handbuch der mikroskopischen Anatomie des Menschen, *Berlin*, vol. vi, pt. 1, p. 282.

HELLMAN, T., AND WHITE, G. . 1930. *Arch. path. Anat.*, cclxxviii, 221.

HEUDORFER, K. . . . . 1921. *Z. ges. Anat.*, I Abt., lxi, 385.

HUECK, W. . . . . . 1927. *Verh. dtsch. path. Ges.*, xxii, 238.

,, ... 1928. *Ibid.*, xxiii, 6.

JÄGER, E. . . . . . 1928-29. *Z. Zellforsch.*, viii, 578.

JOLLY, J. . . . . . 1923. Traité technique d'hématologie, *Paris*, vol. ii, p. 706.

KELVIN, LORD . . . . 1904. Baltimore Lectures, *Cambridge*, p. 615.

KLING, C. A. . . . . 1904. *Arch. mikr. Anat.*, lxiii, 575.

KOPSCH, F. . . . . . 1922. Allgemeine Lymphgefässlehre, *in* Rauber and Kopsch's Lehrbuch und Atlas der Anatomie des Menschen, 10th ed., *Leipzig*, vol. iii, p. 453.

KRUMBHAAR, E. B. . . . . 1942. Lymphatic tissue, *in* Cowdry's Problems of ageing, 2nd ed., *Baltimore*, p. 148.

LABBÉ, M. . . . . . 1898. *C.R. Soc. Biol.*, Paris, x, 1056.

LEVINSTEIN, O. . . . . 1909. *Arch. Laryngol. u. Rhinol.*, Berlin, xxii, 104.

MALLORY, F. B., AND PARKER, F., JR. 1927. *Amer. J. Path.*, iii, 515.

MAXIMOW, A. A. . . . . 1927. Bindegewebe und Blutbildende Gewebe, *in* von Möllendorff's Handbuch der mikroskopischen Anatomie des Menschen, *Berlin*, vol. ii, pt. 1, p. 360.

,, ... 1932. The lymphocytes and plasma cells, *in* Cowdry's Special cytology, 2nd ed., *New York*, vol. ii, p. 603.

MELNIKOW-RASWEDENKOW, N. . 1899. *Beitr. path. Anat.*, xxvi, 546.

MILLER, R. E. . . . . 1932. *Arch. Path.*, xiii, 367.

MILLER, W. S. . . . . 1924. *Amer. Rev. Tuberc.*, ix, 519.

NAGOYA, C. . . . . . 1913. *Frankf. Z. Path.*, xiv, 106.

NORDMANN, M. . . . . 1928. *Arch. path. Anat.*, cclxvii, 158.

OELLER, H. . . . . . 1928. Lymphdrüsen und lymphatisches System, *in* Bethe and Bergmann's Handbuch der normalen und pathologischen Physiologie, vol. vi, pt. 2, p. 995.

ORSÓS, F. . . . . . 1926. *Beitr. path. Anat.*, lxxv, 15.

PARODI, U. . . . . . 1927. *Hæmatologica*, viii, 1.

RENN, P. . . . . . 1912. *Beitr. path. Anat.*, liii, 1.

RICHTER, J. . . . . 1902. *Arch. mikr. Anat.*, lx, 469.

RÖHLICH, K. . . . . 1928. *Z. mikr.-anat. Forsch.*, xii, 254.

ROSSLE, R.	1923 24.	*Ergebn. allg. Path. u. path. Anat.,* xv, Abt. II, 369.
ROSSLE, R , AND YOSHIDA, T.	1909.	*Beitr. path. Anat.,* xlv, 110
ROTTER, W.	1927.	*Arch. path. Anat ,* cclxv, 596.
SCHMELZER, W.	1936.	*Z. Zellforsch.,* xxiv, 303.
SJÖVALL, A., AND SJÖVALL, H.	1930.	*Arch. path. Anat.,* cclxxviii, 258.
STERNBERG, C.	1926.	Die Lymphknoten, in Henke-Lubarsch's Handbuch der speziellen pathologischen Anatomie und Histologie, *Berlin,* vol. i, pt. 1, p. 249.
STILES, H. J.	1892 93.	*Edinb. Med. J.,* xxxviii, 26.
TEICHMANN, L.	1861.	Das Saugadersystem vom anatomischen Standpunkte, *Leipzig,* p. 27.
THOMÉ, R.	1902.	*Z. Naturw.,* xxxvii, 133.
THOMPSON, D'ARCY W.	1942.	On growth and form, new ed., *Cambridge,* p. 708.
TOLDT, C.	1888.	Lehrbuch der Gewebelehre, 3rd ed , *Stuttgart,* p 383.
UCHINO, S.	1925.	*Beitr. path. Anat.,* lxxiv, 405.
WETZEL, G.	1926.	*Klin. W'schr ,* v, 1587.
„	1938.	Die blutbildenden Organe, in Peter, Wetzel and Heiderich's Handbuch der Anatomie des Kindes, vol. i, pt. 1, p. 140.

THE EFFECT OF FOSTER-NURSING ON THE MORPHOLOGY OF THE MAMMARY GLANDS IN MICE

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(Plates LXXXVII XC)

Cancer of the breast appears spontaneously in some laboratory mice, and in a number of institutions genetically homozygous strains of mice have been bred, differing among other things in the frequency with which they develop mammary carcinoma These animals have been studied for the purpose of determining the role of several factors in the induction of new growth and certain general inferences have been drawn

1 The genetic constitution of an animal determines to some extent its liability to develop breast cancer (hereditary susceptibility), but this liability is slight in the absence of extrachromosomal factors

2 Extrachromosomal factors play a dominant role in the susceptibility of mice to breast cancer These factors are transmitted through the cytoplasm of the ovum (van Guhk and Korteweg, 1940) or in the milk on which the young are reared

3 The spontaneous development of such cancer depends on the existence of a suitable humoral environment in the animal It also depends on the responsiveness of the tissues to products of metabolism, hormones, and their interactions

The mammary glands in mice show mild cyclic changes in adults and marked changes attendant on pregnancy and lactation in breeding females The alteration in breast tissue during the evolution of cancer shows three stages There is a phase of resistance to growth and a phase of preparatory growth prior to the phase of cancer formation The relative dominance of any one phase or the rapidity of progress from one stage to the next varies in different strains of mice and is believed to be correlated with its susceptibility (Loeb and Suntzeff, 1941)

Recent investigations have been directed towards a study of the physical and chemical characters and the distribution in the viscera of the milk borne factor which has been shown to be present in the

milk of certain strains of mice. The tumour-inducing agent in the milk possesses properties usually associated with viruses or cytoplasmic and molecular determinants (Bryan *et al.*, 1941-42).

It was thought that the relative significance of the three factors— the presence of a transmissible tumour-inducing agent, a disturbed hormonal equilibrium and a genetic susceptibility—might be elucidated by a careful study of the morphology of mammary glands under controlled experimental conditions in cancer-susceptible and cancer-insusceptible strains of mice. The morphological appearances of breasts in healthy virgin mice belonging to four strains at four age periods were therefore studied and have already been reported (Ranadive, 1945). The breasts in virgin females of these four strains exhibited clearly recognisable differences in ductal pattern and acinar arrangement at 6-8 months. The ductal pattern was identical in the ten breasts of the same animal. An attempt is now made to describe the effect of foster nursing on the microscopic structure of breasts in three strains between the ages of 7 and 8 months. Preliminary studies of this nature have been referred to by van Gulik and Korteweg (1940), Shimkin *et al.* (1941-42), Bittner *et al.* (1944) and Bittner and Huseby (1946).

<div align="center">MATERIAL AND METHODS</div>

The experimental animals were descendants of mice kindly presented to us in the *U.S.A.* The strains C_{57} Black and C_3H were from the Roscoe B. Jackson Laboratories and strain Strong A from the Department of Anatomy, Yale University. The stocks were maintained by brother-sister mating between young healthy animals. They were in their F10-11 generations since their arrival in India. For purposes of foster-nursing half or whole litters were exchanged within 5-15 hours after birth. After weaning, the males and females were separated, and litter mates were kept under identical conditions for about six months. Vaginal smears were examined daily from soon after the seventh month and the animals were killed during full œstrus. The technique for the preparation of whole mounts of breasts has been previously described (Ranadive) and does not differ in any essential respect from that suggested by Gardner *et al.* (1939). Except in a few instances the ten breasts of all the animals were studied and the characters of the ductal pattern and acinar arrangement recorded. '

Table I shows the number of virgin mice from the three strains and the number of mammary glands which were studied in each group. The figures regarding tumour frequency are based on information supplied by their laboratories of origin or published in the literature. The incidence may have altered to some extent during the sojourn of the strains in this country (Bittner, 1941).

An examination of 1711 entire breasts from 173 mice showed that the arrangement of ductal branching could be separated into three distinct patterns and the grouping of acini along the ducts into three types. It was surprising that although the mammary response to œstradiol implants varied to a certain extent in different breasts of the same animal, the ductal pattern and acinar arrangement in the ten breasts of any animal were markedly similar after foster-nursing. The ductal patterns designated α, β and γ and the acinar arrangements A, B and C are described first to avoid frequent repetition later.

<div align="center">

TABLE I

Numbers of animals examined, showing the known incidence of breast cancer in the experimental and control groups

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Nursed by strain	No of virgins surviving after 7 months	No of mammary glands studied	Published Incidence of breast cancer	Author
			Highly susceptible strain C₃H	
C₃H	20	200	95 100 per cent virgins and breeding females	Andervont (1938)
C₅₇ Black	20	196	46 0 per cent breeding females	Andervont and McEleney (1939)
Strong A	20	200	94 per cent	Heston and Andervont (1943 44)
			Non-susceptible strain C₅₇ Black	
C₅₇ Black	20	200	0 5 per cent breeding females	Andervont (1938)
C₃H	20	200	9 0 per cent breeding females	Andervont and McEleney (1939)
Strong A	20	200	20 0 per cent breeding females	Deome (1940)
			Susceptible strain Strong A	
Strong A	20	194	4 9 per cent virgins 83 6 per cent breeding females	Bittner (1939)
C₅₇ Black	20	196	4 9 per cent breeding females	Bittner (1937)
C₃H	13	125	16 0 per cent	Heston and Andervont (1943 44)

Duct patterns

(α) In this pattern the mammary ducts are long and slender. Branching proceeds to the third or fourth order. Side branches from the main ducts are few and short. The pattern assumes the character of an open network against a homogeneous stroma. This is the commonest pattern encountered in the present material (fig 1)

(β) The main ducts and the order of ductal branching are similar to those in pattern α. There are, however, a large number of short side branches from the main ducts, so that the general appearance is of a profusely branching comparatively complicated closed ductal network (fig 2). There is an occasional variant of this type (β) in which the main ducts are peculiarly dilated. They branch and re branch along the whole extent of their length, so that the secondary and tertiary branches appear as thin thread like processes which ramify profusely in a dense periductal stroma (fig. 3). The network is closely woven, simulating the ductal proliferation during pregnancy. This pattern occurs very rarely and is not peculiar to any particular strain or to any group after foster nursing.

(γ) The characteristic of this type is a wide dilatation of the main duct and its secondary offshoots. They are about 3 or 4 times as wide as the

corresponding ducts in the first pattern (α). The secondary branches from the main ducts are few, short and stunted, with blunt ends or rounded tips (fig. 4).

Acinar arrangement

(A). This arrangement is characterised by an almost complete absence of single acinar buds or groups of acini. When this arrangement is associated with the ductal pattern α the mammary gland simulates the outlines of a bare tree (fig. 5).

(B). Small acinar buds in groups of 4-10 alveoli are dotted along the entire extent of the gland. In some glands these groups may be restricted to isolated areas. The appearance of the mammary gland under low magnification with the buds stained bright crimson by "Kernechtrot" could be likened to a tree in bud (fig. 6).

(C). There is an uneven *multiplication of acinar buds* in this type which leads to the formation of irregular clusters of acinar cells (fig. 7). The localised clusters are seen as opaque nodular masses attached to the ductal branches and have been described as "focal acinar clusters" or "hyperplastic nodules". Such nodules, usually few in any gland, are produced by focal abnormal growth of acinar epithelial cells and have been regarded as precancerous lesions (Taylor and Waltman, 1940 ; Shimkin *et al.*, 1941-42). An occasional variant of type C (C') consists of small acinar buds evenly distributed along the ducts as in B, with some buds flowering into big hyperplastic nodules in isolated spots. The appearances in a stained preparation resemble the blossoms of red cassia in spring (fig. 8).

EXPERIMENTAL FINDINGS

Table II indicates the changes observed in the architecture of mammary glands after foster-nursing.

Series I

(*a*) Twenty C_3H virgins nursed by their mothers were used as controls for this group : 15 showed simple ductal branching with few slender offshoots, 13 showed hyperplastic nodules (fig. 9) in almost all ten mammary glands of each animal, the number of nodules ranging from three to as many as twelve in the large thoracic pair. One of these females developed a palpable tumour (adenoma) in one breast.

(*b*) In the C_3H virgins nursed on non-susceptible C_{57} Black mothers no change in ductal pattern was noticed from that seen in the control animals. There was, however, a notable absence of hyperplastic nodules in 18 out of the 20 animals (fig. 10). The two remaining virgins each developed a single hyperplastic nodule in one out of the ten mammary glands.

(*c*) Of the C_3H females suckled by susceptible Strong A about half (9/20) showed an appreciable change in ductal pattern from the simple (α) to the complex (β) type. Almost two-thirds (14/20) were devoid of any acinar structure (fig. 11) and in only two of them were hyperplastic nodules encountered. This is in marked contrast with their incidence in the control animals (13/20).

EFFECT OF FOSTER-NURSING ON MOUSE BREAST
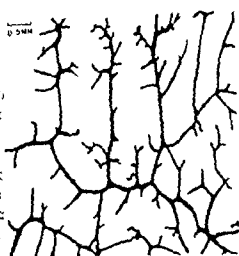
Fig. 1 —Pattern α, showing simple ductal branching with few side off-shoots.

Fig 2 —Pattern β, showing complex ductal branching with large number of side off-shoots

Fig 3 —Pattern β' An occasional variant of type β with main ducts slightly dilated and a dense network of thin threadlike side branches
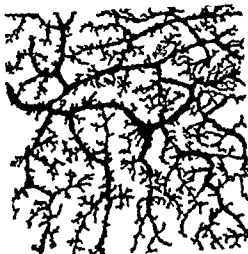
Fig 4 —Pattern γ Main ducts are big and dilated with few short blunt side offshoots

Fig 5 —Type A Complete absence of acinar structure is the characteristic of this type
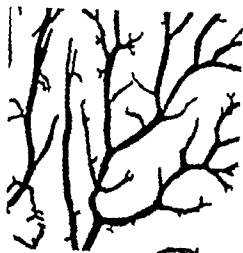
Fig 6 —Type B Small groups of 4 to 10 acini are seen uniformly placed along the ductal branches.
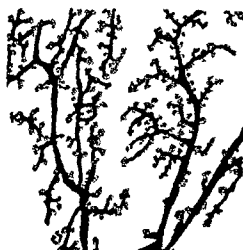
Fig. 7 —Type C. Big localised hyperplastic nodules appearing like dark opaque masses are attached to long slender ductal branches.

Fig 8 —Type C'. An occasional variant of type C Small acinar buds appear uniformly distributed along the ducts, some of which open out into definite hyperplastic nodules
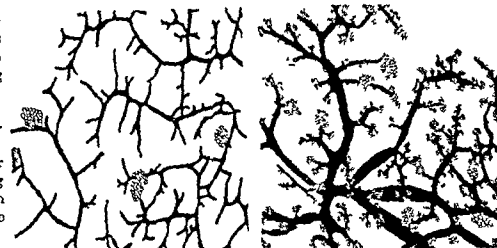
Figs 1-4, ductal patterns, figs 5-8, acinar arrangements, based on camera lucida tracings

Series II

(a) In the 20 C_{57} Black females nursed by their own mothers there was remarkably little variation in the breast pattern. Most of them (18/20) possessed a simple mammary tree with few and short side branches, and were completely devoid of acinar structures (fig. 12). In only 4 animals were small acinar buds seen, but there were no hyperplastic nodules in any of the 20 animals.

TABLE II

Changes observed in the architecture of the mammary glands after foster-nursing

Nursed by strain	Total no. of virgins	Ductal pattern			Acinar arrangement		
		α	β	γ	A	B	C
I. Highly susceptible strain C_3H							
C_3H .	20	15	5	0	6	1	13
C_{57} Black .	20	15	5	0	12	6	2
Strong A .	20	11	9	0	14	4	2
II. Non-susceptible strain C_{57} Black							
C_{57} Black .	20	18	1	1	16	4	0
C_3H .	20	7	3	10	8	3	9
Strong A .	20	15	4	1	10	6	4
III. Susceptible strain Strong A							
Strong A .	20	9	10	1	13	7	0
C_{57} Black .	20	14	6	0	17	2	1
C_3H .	13 *	10	3	0	4	0	9

* Twenty-four strong A females were transferred to C_3H nurses. Mortality among these was so high that only 13 females survived for the seven-month period.

(b) Of C_{57} Black virgins nursed on the milk of the susceptible C_3H strain, one-half (10/20) presented a peculiar dilatation of mammary ducts (γ), the significance of which is discussed later. Hyperplastic nodules were encountered in most of the breasts of about half the animals (9/20) (fig. 13).

(c) The C_{57} Black females fostered by Strong A nurses showed no significant alteration in the duct pattern. There was, however, a noticeable change in the acinar arrangement. Whereas none of the control virgins (C_{57}/C_{57}) showed any acinar structures at seven months, half the animals suckled on Strong A milk presented these structures (6+4/20). Six out of these ten animals showed uniformly distributed acinar buds along the ductal branches; the other four showed well-developed hyperplastic nodules in addition (fig. 14). One of these four animals disclosed an interesting morphological pattern. Localised

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Fig. 9.

Fig. 10.

Fig. 11.

Fig. 12.

Fig. 13.

Fig. 14.

PLATE LXXXIX

FIGS. 15-17.—Photomicrographs showing the effect of foster-nursing on the susceptible strain Strong A.

FIG. 15.—Portion of an entire breast of a Strong A virgin nursed by her mother. The ductal pattern is the complex (β) type with many side branches comparable to that in fig. 11.

FIG. 16.—Part of an entire breast of a Strong A virgin nursed on a non-susceptible C_{57} Black. The ductal branches are all bare and without acini. The pattern simulates α A type in normal C_{57} (fig. 12).

FIG. 17.—Portion of an entire breast of a Strong A virgin fostered on the highly susceptible strain C_3H. One focal hyperplastic nodule is seen. The ductal arrangement in this particular animal is complex (β).

FIG. 18.—Camera lucida drawing of a whole mount preparation from the major portion of the third thoracic mammary gland of a C_{57} virgin fostered on Strong A. Main ductal tree is very plain (α). On it is superposed the complex ductal network of type β' and in its centre hyperplastic nodules are seen.

FIG. 19.—Microphotograph taken from the central portion of the preparation drawn in fig. 18. These are complex ducts of type β' with hyperplastic nodules in the centre.

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Fig. 15.

Fig. 16.
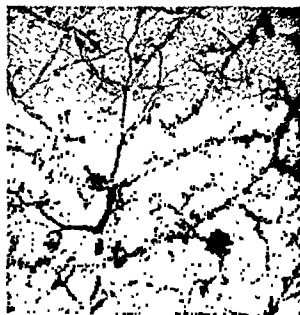
Fig. 17.
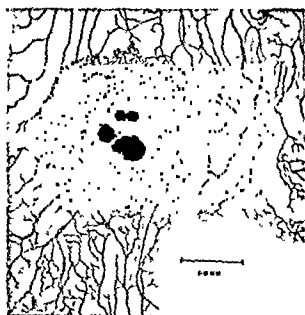
Fig. 18.
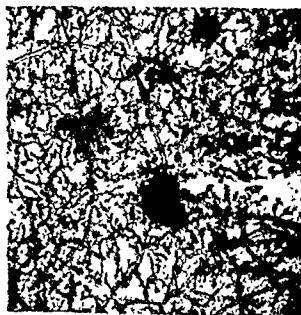
Fig. 19.

susceptible strain and in a few fostered on milk of the susceptible strain Strong A.

(a) Out of nine Strong A males fostered on C_{57} Black, eight developed breasts completely devoid of hyperplastic nodules. Only one animal showed a few indistinct nodules in two out of the ten mammary glands (fig. 20).

(b) Males of strain Strong A transferred to C_3H nurses developed a dilated and stunted ductal system in almost all the animals, and big hyperplastic nodules covered most of the ducts throughout the extent of the glands (fig. 21).

(c) Non-susceptible C_{57} males did not show any breast development when nursed on their own milk, except for a couple of rudimentary ducts.

(d) The males of C_{57} Black, on the other hand, when suckled by " A " mothers, produced big hyperplastic nodules in the breasts of eight out of twelve animals (fig. 22).

DISCUSSION

A study of the material described above indicates certain general characteristics regarding the morphological alteration of the mammary glands after foster-nursing. The architecture of these glands alters in the direction of the structure prevalent in the foster-mother. The alteration affects the ductal pattern as well as the acinar arrangement.

The numerical data relating to the acinar arrangements in the six groups of foster-nursed animals have been compared with the data for the control groups (table II) and the statistical significance of the differences assessed (table III).

TABLE III

Statistical analysis of the data presented in table II

Series	Foster-nursed group	Control group	Value of χ^2	Range of P
1	C_3H/C_{57}	C_3H/C_3H	13·636	0·01 to 0·001
2	C_{57}/C_3H	C_{57}/C_{57}	11·809	0·01 ,, 0 001
3	C_3H/A	C_3H/C_3H	13·066	0·01 ,, 0·001
4	A/C_3H	A/A	17·817	0·001 ,, 0·0001
5	C_{57}/A	C_{57}/C_{57}	5·794	0·1 ,, 0·05
6	A/C_{57}	A/A	4·311	0·1 ,, 0·05

(i) The values of χ^2 in series 1-4 are significant, while in series 5 and 6 they fall near the line of significance. This last result, however, may depend on the fact that the quantitative differences in the control groups also are not marked.

(ii) The data relating to the acinar arrangements in the breasts of the foster-nursed animals and their foster-mothers, when analysed in the same manner, show no significant difference, *suggesting an*

alteration of the acinar arrangement in the fostered animals in the direction of the foster-mothers'.

(iii) The numerical data of the ductal pattern prevalent in the three strains C₃H, C₅₇ Black and Strong A have not been subjected to statistical treatment, as the differences are not essentially quantitative in nature.

Alterations in the ductal pattern. About half the C₃H mice after foster-nursing by Strong A females show the more profuse ductal branching and comparatively complicated network characteristic of strain Strong A (fig. 11), and a majority of the Strong A mice nursed by C₃H mothers exhibit the simple (α) pattern usually encountered in the foster-mothers (fig. 17). The effect on the ducts is masked in those animals where the pattern is similar in the mothers and foster-mothers (fig. 10). Thus, as both C₃H and C₅₇ Black show an analogous pattern (α), no evident change could be expected after reciprocal foster-nursing. However, in C₅₇ females nursed on C₃H almost half the animals show a peculiar dilatation of ducts with blunt side offshoots (γ) (fig. 13). A similar change was referred to by Gardner (1939) prior to the development of cancer in œstrogen-treated animals.

Alterations in acinar arrangement. As regards the acinar arrangement it was observed that hardly any C₃H or Strong A females foster-nursed by C₅₇ show hyperplastic nodules (figs. 10 and 16), and only infrequently develop even small acinar groups. This supports the observation of Bittner *et al.* that only " occasionally a nodule " and " not more than one to a gland " was found even in breeding females of C₂H and Strong A fostered on non-susceptible mice. On the other hand, it was found that a majority of C₅₇ females nursed on C₃H or Strong A develop a very varied arrangement of acinar clusters (figs. 13 and 14). If these nodules can be accepted as precursors of cancer as suggested by several investigators (Taylor and Waltman, Shimkin *et al.*), then the observations described above may be taken as affording a morphological basis for the shift in the incidence of breast cancer in mice after foster-nursing. It may be pointed out that in C₅₇ fostered on dba (van Gulik and Korteweg) and in C on C₃H (Shimkin *et al.*) hyperplastic nodules were observed in the mammary glands under similar conditions.

Foster-nursed males with intact testes when treated with œstradiol exhibited a similar alteration in the morphological pattern of mammary glands. Males of the cancer-susceptible strain Strong A, when nursed by non-susceptible C₅₇ Black and treated with œstradiol, rarely showed hyperplastic nodules in the breasts (fig. 20), whereas all the males of the same strain when nursed by mice of the susceptible strain C₃H and similarly treated developed hyperplastic nodules (fig. 21). So also intact males of the non-susceptible strain C₅₇ Black, nursed by their own mothers and treated with a large dose of œstradiol, rarely showed any breast development; but when they were nursed on the

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FIG 20 —Entire mammary gland of a Strong A male fostered on the non susceptible C_{57} Black. The ductal pattern is very simple without acini
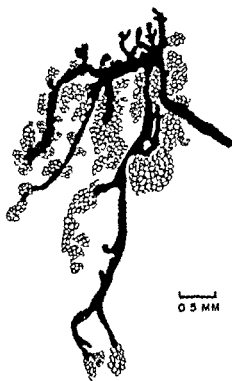
FIG 21 —Entire mammary gland of a Strong A male nursed on the highly susceptible strain C_3H. The ducts are dilated, with few side branches. Big hyperplastic nodules cover part of the ducts

FIG 22 —Entire mammary gland of a C_{57} Black male fostered on Strong A. The ducts are slender and the mammary gland is much better developed. Big hyperplastic nodules cover the ducts all over the gland

FIGs 20 22 —Camera lucida tracings of entire mammary glands of foster nursed œstrogen treated males

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susceptible strain Strong A and similarly treated they showed hyperplastic nodules in a large majority of the animals (8/12) (fig. 22). Hyperplastic nodules rarely occurred in spite of the administration of an adequate amount of œstradiol in the absence of active milkborne factor. With an identical dose of œstradiol the same strain displayed a significant difference in the breast pattern after a change of the milk-borne factor. The animals thus provided a satisfactory control to the observations on foster-nursed females of the same strain. Twombly (1940), Bonser (1944) and Dmochowski and Gye (1944) noticed a corresponding change in the incidence of breast cancer in intact foster-nursed males after œstrogen treatment. With œstrogen administration Twombly recorded an incidence of 33 per cent. breast tumours in C_{57} males after feeding on the milk of R III (highly susceptible) nurses, an incidence which had not been previously observed in similar animals nursed by their own mothers.

The observations recorded above suggest that there is a difference, either quantitative or qualitative, in the milk-borne factor of the three strains of mice. Most of the animals from the three strains develop hyperplastic nodules in their breasts when they are nursed by C_3H females. On the other hand, the milk-borne factor of the susceptible strain Strong A is able to induce abnormal growth in some animals of the non-susceptible strain C_{57} only and not in Strong A or C_3H. The milk of C_{57} Black fails to induce hyperplastic nodules in any of the animals used in this experiment. The milk-borne factor of the three strains thus appears to vary in its quality or quantity. However it is not only the milk-borne factor of the nurse but the genetic constitution of the nursling and the responsiveness of the breast tissue to the particular milk-borne factor which ultimately determine the breast pattern of the animal. Dmochowski (1945) has recently reported a similar difference in the potency of tumourinducing substance obtained from splenic and dried tumour tissue in three strains of mice with different genetic constitution.

Mammary cancer develops frequently in C_3H virgins (95 per cent.) but rarely in Strong A virgins (4-5 per cent.). The difference in tumour incidence between virgins of C_3H and Strong A and between virgin and breeding females of Strong A strain is explained by Bittner *et al.* (1944) as due to a lack of adequate hormonal stimulation in Strong A virgins. An increased production of ovarian hormones during pregnancy results in a marked increase of breast tumour incidence (80-85 per cent.) in breeding females of Strong A strain. A similar change is indicated by the presence of precancerous nodules at 7-8 months in the breasts of Strong A virgins nursed by C_3H mothers. It can therefore be assumed that the milk substance in C_3H mice provides Strong A virgins with all the factors needed for inducing abnormal proliferation of the glandular tissue.

These observations afford interesting material for speculation regarding the action of the milk-borne factor. It is unnecessary to

elaborate at this stage our ideas regarding the nature of this substance and its tumour-inducing properties. It is possible that the milk-borne factor acts on the mammary glands through its effect on the endocrine system, but this would not appear to be the only avenue of its activity, because it is not possible to induce tumours in all strains of mice by increased hormonal stimulation. It has been shown by several investigators that large doses of œstrogens are unable to induce abnormal mammary gland proliferation without the mediation of an active milk-borne factor. The presence of this substance has been demonstrated in the breast parenchyma of certain strains of mice as well as in their mammary tumours (Bryan *et al.*). It therefore appears that malignant mammary proliferation can only be induced by a combined action of the milk-borne factor on the breast parenchyma and on the endocrine glands. Such proliferation fails to materialise in the absence of this substance in the milk or an inadequate response to its presence by the endocrine glands.

SUMMARY

1. There is a noticeable difference in the structure of the mammary glands of virgin mice at 7-8 months in the three strains C_3H, Strong A and C_{57} Black.

2. Foster-nursing on animals of another strain alters the morphology of the breast in these mice. If there is a difference in the pattern of the ducts between the nurse and the foster-nursed strain, a change is effected in the ductal pattern of the nursling. The acinar arrangement was altered in all six groups of foster-nursed animals. The architecture of the mammary gland of foster-nursed females is altered in the direction of the structure prevailing in the foster-mother. This is clearly demonstrated in C_3H mice nursed on C_{57} Black and in C_3H nursed on Strong A, as well as in Strong A nursed on C_{57} Black and Strong A nursed on C_3H. A statistical analysis of the numerical data relating to the foster-nursing of female mice confirms these conclusions.

3. The effect of foster-nursing on œstrogen-treated intact male mice is analogous to that observed in females of the same strain.

REFERENCES

ANDERVONT, H. B. .   .   .   .   .   .   1938.   Publ. Hlth. Rep., *Washington*, liii, 1647.

ANDERVONT,   H.   B.,   AND   1939.   *Ibid.*, liv, 1597.
    McELENEY, W. J.

BITTNER, J J   1937   *Amer J Cancer*, xxx, 530

"   1939   *Publ Hlth Rep* *Washington*, liv, 1113

"   1941   *Cancer Research* i, 115

BITTNER J J , AND HUSEBY, R A   1946   *Ibid* , vi, 235

BITTNER, J J , HUSEBY, R A , VISSCHER, M B , BALL, ZELDA B , AND SMITH, FERN   1944   *Science*, xcix, 83

BONSER, GEORGIANA M   1944   *This Journal*, lvi, 15

BRYAN, W R , KAHLER, H , SHIMKIN, M B , AND ANDERVONT, H B   1941 42   *J Nat Cancer Inst* , ii, 451

DEOME, K B   1940   *Amer J Cancer*, xl, 231

DMOCHOWSKI, I   1945   *Brit J Exp Path* , xxvi, 267

DMOCHOWSKI, L , AND GYE, W F   1944   *Ibid* , xxv, 115

GARDNER, W U   1939   *Arch Path* , xxviii, 138

GARDNER, W U , STRONG, L C , AND SMITH, G M   1939   *Amer J Cancer*, xxxvii, 510

van GULIK, P J , AND KORTEWEG, R   1940   *Verslagen Nederl Akad van Weten-scheppen*, xliii, 891

HESTON, W E , AND ANDERVONT, H B   1943 44   *J Nat Cancer Inst* , iv, 403

LOEB L , AND SUNTZEFF, V   1941   *Arch Path* , xxxii, 739

RANADIVE, KAMAL   1945   *Proc Indian Acad Sci* , xxii, sect B 18

SHIMKIN, M B , GRADY, H G , AND ANDERVONT, H B   1941 42   *J Nat Cancer Inst* , ii, 65

TAYLOR, H C , JR , AND WALTMAN, C A   1940   *Arch Surg* , xl, 733

TWOMBLY, G H   1940   *Proc Soc Exp Biol and Med* , xliv, 617

THE ADRENAL CORTEX IN ESSENTIAL AND RENAL HYPERTENSION

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THERE are many observations which suggest that in hypertension in man there are associated changes in the adrenal cortex, although there is no general agreement as to the nature or frequency of these changes   The position was reviewed by Fishberg (1939), who considered that adrenal cortical hyperplasia is a prominent feature in essential hypertension although its role is completely obscure. Rinehart *et al.* (1941) found that in essential hypertension there was not only adenomatous cortical hyperplasia but also an increase of the mean adrenal weight.   Dempsey (1942), however, was unable to confirm either of these observations.   In the hypertension of Cushing's syndrome, there is commonly adrenal cortical hyperplasia, and in experimental " clamp " hypertension the adrenal cortex or a hormone produced by it is essential for the maintenance of the hypertension (Goldblatt, 1937-38).

In view of these observations, we decided to obtain further data by paying special attention to the endocrine organs in our routine autopsies on cases of hypertension.   Our interpretation of these data is that in both essential and renal hypertension there is a definite increase in the concentration of lipid in the adrenal cortex.

MATERIAL AND METHODS

Our material was derived from routine autopsies at general hospitals and comprised 55 essential hypertensives, including three in the malignant phase, 15 renal hypertensives and 57 non-hypertensive controls.

We recorded the heart weight and measurements for the assessment of left ventricular hypertrophy and in every case made a histological study of the kidneys, pancreas, liver and adrenals, and usually of other organs, to observe hypertensive arteriolar changes.   In assessing heart weight and blood pressure we applied the criteria of Moritz and Oldt (1937).   Heart weights of 450 g. in males or 350 g in females were taken to indicate hypertension.   Those weighing less than 400 g in males or 300 g. in females were considered non hypertensive. Blood-pressure readings of 160/90 or more were considered hypertensive for any age and no case was accepted as a non hypertensive control if there was a record of a systolic pressure over 140 or a diastolic over 90.

To establish a diagnosis of hypertension, we required two out of the three positive criteria : a high blood pressure record, left ventricular hypertrophy and hypertensive arteriolar changes.   We discarded all cases of valvular disease

of the heart with the exception of one in which there was clinical evidence of prolonged hypertension, and all cases of recent pregnancy, the former because the heart weight was not a reliable criterion of hypertension and the latter because of the physiological hypertrophy of the adrenal cortex in pregnancy. We also discarded cases where the adrenals were the seat of disease, such as tuberculosis or tumour metastasis; needless to say we did not exclude examples of cortical adenoma.

Examination of adrenals

After fixation for 24-48 hours in 10 per cent. formol-saline the adrenals were carefully stripped free of all extra-capsular fat and connective tissue, gently dried by blotting, and weighed. Blocks for histological examination were taken through the centre of one gland, or from both in the event of disparity in their appearance, and the remainder of both glands was cut into small pieces and thoroughly mixed. Approximately 5 g. of this material was accurately weighed, ground up with sand and extracted with absolute alcohol and ether for estimation of the percentage lipid content, and thus of the total lipid in the two glands. We are aware that formaldehyde-precipitated protein protects crystals of cholesterol esters to some extent from solution by lipid solvents, but we found it impossible to strip the extra-capsular tissue adequately before fixation.

Frozen sections were stained with Scharlach R and examined under polarised light. For purposes of visual estimation of the lipid content of the cortex, this was divided into three equal zones: the outer, middle and inner thirds. The content of sudanophil lipid and of anisotropic lipid in each zone was recorded in terms of arbitrary units: 0, $\pm$, 1, 2 or 3. We found no advantage in attempting to estimate the proportion of lipid visible in the three anatomical zones. The sum of the units in all three zones that we chose seemed to give a more accurate expression of the total lipid content than one could obtain by the use of a single figure. In arriving at the sum-total, $\pm$, which indicated a bare trace, was counted as $\frac{1}{4}$.

Assessment of sepsis, nutrition and atheroma

Albrecht and Weltmann (1911) and others have drawn attention to the great reduction in the anisotropic lipid content of the adrenal cortex in infections and this observation has been confirmed. It was therefore necessary to consider this factor lest it should invalidate our conclusions. For this purpose we divided our cases into three groups: those with no sepsis, those with slight or recently acquired sepsis such as a terminal bronchopneumonia, and those with severe or chronic sepsis.

We thought it necessary also to consider the state of nutrition of our subjects and the degree of arterial atheroma, since the latter might be associated with a general disturbance of cholesterol metabolism. In assessing the degree of atheroma the aorta, the coronary and cerebral arteries and the renal and other large arteries were examined and an arbitrary figure awarded: 0, 1, 2 or 3. A few yellow streaks in the aorta were disregarded.

RESULTS

Adrenal weight and essential hypertension

The mean weight of both adrenals in 55 essential hypertensives was 11·33 g. (standard deviation = 2·68) and in 57 non-hypertensive controls 10·94 (S.D. = 2·61). The difference between these is

obviously not significant, a finding which agrees with that of Dempsey. We had no record of the body weight of our subjects, so we could not apply any correction for this factor, and we found that the weight of the adrenals bore no relationship to body length in our non-hypertensive controls.

Nodularity of adrenal cortex and essential hypertension

We found a significant association * between cortical nodularity and essential hypertension (table I).

TABLE I

Cortical nodularity and essential hypertension

Adrenals	Controls	Essential hypertensives
Smooth . . .	24	10
Nodular . . .	18	35
$\chi^2 = 6\cdot4$ $P < 0\cdot02$		

Total and percentage adrenal lipid and essential hypertension

The total lipid in the adrenals was obtained from the product of the adrenal weight and the percentage of lipid. In 52 essential hypertensives the mean total lipid was $0\cdot869$ g. (S.D. $= 0\cdot405$) and in 54 non-hypertensive controls $0\cdot679$ (S.D. $= 0\cdot568$), a difference which is not significant. There is, however, a highly significant correlation between a high percentage of lipid and essential hypertension (table II). The mean percentage lipid for the hypertensives and controls taken together was $7\cdot17$.

TABLE II

Percentage lipid and essential hypertension

	Below mean	Above mean
Controls	38	16
Hypertensives . . .	21	31
$\chi^2 = 8\cdot58$ $P < 0\cdot01$		

The lack of correlation between total lipid and hypertension, in contrast to the highly significant association with percentage lipid,

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* Throughout the text the term "highly significant" indicates a probability (P) of less than 0 01, the term "significant" a probability of between 0·05 and 0·01. A probability of over 0·05 is regarded as not significant. Yates's correction has been applied where necessary.

is to be explained as due to the considerable variation in adrenal weight, which we have shown to bear no significant relation to hypertension.

Adrenal sudanophil lipid and essential hypertension

We found a highly significant relationship between the sudanophil lipid content, gauged by visual estimation of a stained section, and essential hypertension (table III).

TABLE III

Adrenal sudanophil lipid and essential hypertension

	Sudanophil units	
	0-4	5-9
Controls	37	17
Hypertensives . . .	22	30
$\chi^2 = 10.07$　　P$<$0.01		

Anisotropic cortical lipid and essential hypertension

In 41 out of 53 cases of essential hypertension the adrenal cortex contained from 3 to 9 units of anisotropic lipid, while in the non-hypertensive controls only 15 out of 54 contained as much (table IV). This greater incidence of a high content of anisotropic lipid is statistically highly significant.

TABLE IV

Anisotropic lipid and essential hypertension

	Anisotropic lipid units	
	0-2$\frac{2}{3}$	3-9
Controls	39	15
Hypertensives . . .	12	41
$\chi^2 = 24.28$　　P$<$0.01		

Renal hypertension and adrenal cortical lipid

Only 15 cases of post-nephritic hypertension were available for study and the number is insufficient to do more than indicate that adrenal cortical lipid shows a tendency to be increased in renal as in

essential hypertension. The percentage lipid showed no relationship and the sudanophil lipid was not significantly increased, but a highly significant association was found in the case of anisotropic lipid (table V).

TABLE V

Renal hypertension and adrenal cortical lipid

	Sudanophil units		Anisotropic units	
	0-4	5-9	0-2½	3-9
Controls	37	17	39	15
Renal hypertensives . . .	6	9	1	14
	$\chi^2 = 2.94$ $P < 0.1$		$\chi^2 = 17.13$ $P < 0.01$	

Analysis of results

We are aware that other factors might account for the findings shown in the foregoing tables. It was decided to make a complete analysis of the following five factors : anisotropic lipid, hypertension, age, degree of atheroma and degree of sepsis. The anisotropic lipid factor was chosen since it seemed to be highly correlated with both sudanophil lipid and percentage lipid, so that it would be superfluous to take in all three of these adrenal factors as though they were essentially distinct.

It was decided to ignore the nutrition factor, since the correlation coefficient between nutrition and anisotropic lipid in our sample was less than 0.025 : this is completely insignificant to any level and indicates a complete lack of connection between nutrition and anisotropic lipid in our series.

Each factor was estimated at two levels. Three grades of sepsis were originally recognised—complete absence, moderate and severe. Since it was impossible to attach numerical values to these three grades we made two independent analyses, in the first of which the cases of moderate sepsis were grouped with those of total absence, and in the second the moderate were grouped with the cases of severe sepsis. We may call these analyses A and B respectively.

We denote the variates by suffixes 1-5, where $1 =$ anisotropic lipid, $2 =$ hypertension, $3 =$ age (below or over 50), $4 =$ atheroma, and $5 =$ sepsis.

Analysis A. The ten correlations between the five variates gave the results :

$$r_{12} = 0.4963, r_{13} = 0.3805, r_{14} = 0.2781, r_{15} = -0.3072,$$
$$r_{23} = 0.3856, r_{24} = 0.5542, r_{25} = -0.3604, r_{34} = 0.3449,$$
$$r_{35} = -0.2456, r_{45} = -0.2513.$$

These ten correlation coefficients are all significant to the 1 per cent. level, but in this form it is not possible to declare, for example, that the association between 1 and 2—anisotropic lipid and hypertension—expressed by the coefficient 0·4963 is really an association between these two variates and is not masked or augmented by the interaction of the other three factors. To settle this point we used the technique of statistical elimination to derive a single coefficient expressing the amount of correlation between hypertension and anisotropic lipid when the effects of the other three factors are controlled. Using a capital letter for the final coefficient we found that $R_{12} = 0·3475$.

Analysis B. The ten correlation coefficients in this analysis differ from those in analysis A only where those involving variate 5 are concerned, the results being :

$$r_{15} = -0·4392, \quad r_{25} = -0·3926, \quad r_{35} = -0·1489, \quad r_{45} = -0·2897.$$

Effecting the elimination in this case we found $R_{12} = 0·3021$. It is seen that in both cases R_{12} is bigger than 0·3 and this value is significant beyond the 1 per cent. level. This implies that the connection between hypertension and anisotropic lipid is a real one and not an apparent connection caused by the interaction of the three other characters.

Our figures for the correlation coefficients enabled us to explore the association between hypertension and atheroma, eliminating the other factors. In analysis A the final coefficient $R_{24} = 0·4468$; and in analysis B it is 0·4357. This indicates a real and strong connection between hypertension and atheroma, independent of the other factors.

DISCUSSION

Our results indicate that the proportion of adrenal cortical lipid, including anisotropic lipid, is increased in both essential and renal hypertension. The interpretation of this finding can only be tentative because an increase in lipid *per se* does not prove that there is altered adrenal function. Altered structure in the body so commonly reflects altered function, however, that it is reasonable to take the view that the increased cortical lipid is an indication of altered cortical endocrine activity. This interpretation is supported by and consistent with the known facts relating adrenal cortical activity to the blood pressure.

It is not yet known how the adrenal cortical hormones are formed in the body, but it is well known that many of them are steroid in structure. The amounts, however, are not sufficient to show in section (Kendall, personal communication to Lichtman and McDonald. 1946, p. 78), and our methods of analysis were calculated only to demonstrate gross changes in cortical lipid content, since it was considered that the cortical lipids might reasonably be related to the elaboration of the steroid hormones and that if gross changes were found to be present they would be a strong indication of a disturbance

in the elaboration of these hormones. The sudanophil staining method appeared to be of particular value because the distribution of ketosteroids and cholesterol corresponds closely with the intensity of sudanophil material in the human adrenal cortex (Sarason, 1943).

The possible association in man between abnormal cortical steroid production and hypertension is supported by the evidence of the pressor effects of desoxycorticosterone and other related steroids. Although in man there are no known ill effects of overdosage with natural cortical extracts, the synthetic steroid desoxycorticosterone acetate, which restores the blood pressure and maintains life in Addison's disease, produces hypertension when given in excess, both in Addison's disease (Ferrebee *et al.*, 1939) and in patients without disease of the adrenals (Perera *et al.*, 1944). In rats, desoxycorticosterone and many related steroids have a pressor effect (Grollman *et al.*, 1940). This hypertensive effect of desoxycorticosterone is highly specific and was found by Perera to be independent of the retention of sodium ions or of an increase in the circulating blood volume. Soffer (1946), while recognising that the mode of action of this substance is still highly speculative, makes the interesting suggestion that it might play some part in the metabolism of the kidneys and thus affect the production of the renal hypertensive factor.

There is a striking contrast between the changes in Addison's disease, in which there is loss of adrenal cortical secretion, low blood pressure, inability to retain sodium ions, diminished efferent glomerular arteriolar tone and lowered glomerular filtration pressure, and the changes in essential hypertension, in which there is an increase in cortical lipid, high blood pressure, increased efferent glomerular arteriolar tone and relatively raised glomerular filtration pressure. In Addison's disease the relative decrease in glomerular filtration pressure persists even when a normal blood pressure is maintained by desoxycorticosterone or supplementary sodium chloride therapy (Talbott *et al.*, 1942). In essential hypertension increased tone of the efferent glomerular arterioles is one of the early abnormal functional changes in the kidneys (Goldring and Chasis, 1944).

These pathological conditions of hypotension and hypertension emphasise the association between the adrenal cortex, the efferent glomerular arteriolar tone and the blood pressure. It seems more than a possibility that the adrenal cortex can act as a guardian of the glomerular filtrate by producing an increase in efferent glomerular arteriolar tone, the general blood pressure being geared to this increase of tone so that in the glomeruli the blood flow and filtrate are preserved despite the efferent arteriolar constriction. How this control is exercised is not clear. There is some evidence, however, that the pressor agent renin is released from the afibrillar cells of the afferent glomerular arterioles (Goormaghtigh, 1939); there is thus a gearing pressor agent conveniently placed to produce its greatest pressor effect on the efferent glomerular arterioles and a smaller

but widely dispersed pressor effect on the general systemic arterioles. It is feasible that the adrenal cortex in its role of safeguarding the glomerular filtrate acts through this pressor agency, either by increasing the production of renal pressor substance or by increasing the arteriolar reaction to this substance. It is generally accepted that the adrenal cortex is an important guardian of the sodium content of the internal environment, especially by the control it exercises on renal tubular activity. This control, obviously of fundamental importance to the organism, can only be effective in the presence of an adequate glomerular filtrate. The possible role of the adrenal cortex in safeguarding the adequacy of this filtrate is therefore a role which is complementary and adjuvant to its vital sodium-regulating activity.

The concept of a normal adrenal cortical pressor mechanism directed to the maintenance of the glomerular filtrate and perhaps correlated with the renal tubular sodium-conserving process is tentative, but it appears to be consistent with the known disturbances in disease. These disturbances in essential hypertension include an increase in adrenal cortical lipid, in efferent glomerular arteriolar tone, and in the systemic blood pressure. The present concept suggests that these disturbances in essential hypertension may reflect variation in a single normal process by which the adrenal cortex maintains the glomerular filtrate.

SUMMARY

1. A study was made of the adrenals in 55 cases of essential hypertension, 15 cases of renal (post-nephritic) hypertension and 57 non-hypertensive controls coming to autopsy.

2. In both essential and renal hypertension there was a significant increase in adrenal cortical lipid.

3. The possible significance of these findings in the pathogenesis of essential hypertension is discussed and it is suggested that they may all reflect variation in a single normal process by which the adrenal cortex maintains the glomerular filtrate.

REFERENCES

ALBRECHT, H., AND WELTMANN, O.   1911.   Wien. klin. Wschr., xxiv, 483.

DEMPSEY, W. S.   1942.   Arch. Path., xxxiv, 1031.

FERREBEE, J. W., RAGAN, C., ATCHLEY, D. W., AND LOEB, R. F.   1939.   J. Amer. Med. Assoc., cxiii, 1725.

FISHBERG, A. M.   1939.   Hypertension and nephritis, 4th ed., Philadelphia, p. 583.

GOLDBLATT, H.   1937-38.   Ann. Int. Med., xi, 69.

GOLDRING, W., AND CHASIS, H.   1944.   Hypertension and hypertensive disease, London, p. 55.

GOORMAGHTIGH, N. . . . . 1939. *Brux. méd.,* xix, 1541.

GROLLMAN, A., HARRISON, T. R., 1940. *J. Pharmacol and Exp. Therap.,* AND WILLIAMS, J. R., JR.   lxix, 149.

LICHTMAN, A. L., AND McDONALD, 1946. *Arch. Path.,* xlii, 69. J. R.

MORITZ, A. R., AND OLDT, M. R. . 1937. *Amer. J. Path.,* xiii, 679.

PERERA, G. A., KNOWLTON, ABBIE 1944. *J. Amer. Med. Assoc.,* cxxv, 1030. I., LOWELL, ALICE, AND LOEB, R. F.

RINEHART, J. F., WILLIAMS, O. O., 1941. *Arch. Path.,* xxxii, 169. AND CAPPELLER, W. S.

SARASON, E. L. . . . . . . 1943. *Arch. Int. Med.,* lxxi, 702.

SOFFER, L. J. . . . . . . 1946. Diseases of the adrenals, *London.* p. 215.

TALBOTT, J. H., PECORA, L. J., 1942. *J. Clin. Invest.,* xxi, 107. MELVILLE, R. S., AND CONSO- LAZIO, W. V.

HEPATIC ALKALINE PHOSPHATASE: HISTO-LOGICAL AND MICROCHEMICAL STUDIES ON LIVER TISSUE IN NORMAL SUBJECTS AND IN LIVER AND IN BONE DISEASE

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(PLATES XCI-XCVI)

THE increase in serum alkaline phosphatase in obstructive jaundice over that found in primarily parenchymal liver disease has been shown to be of practical diagnostic value (Sherlock, 1946). The mechanism of this change in jaundice is, however, uncertain, and in particular the origin of the phosphatase, whether osseous or hepatic, remains in doubt. Gomori (1939) has described a histological technique for the demonstration of hepatic alkaline phosphatase. It was thought that the application of this method with actual microchemical analysis of hepatic alkaline phosphatase, not only in liver disease, but also in bone disease with raised serum alkaline phosphatase, might provide useful information. Aspiration biopsy specimens were used, as well as hepatic samples obtained at operation or more rarely at autopsy. This enabled us to study the changes occurring during the course of the illness and to make comparison with alterations in the blood level of the enzyme.

METHODS

Liver tissue was obtained by the aspiration biopsy technique previously described (Sherlock, 1945), occasionally at surgical operation. As has been noticed by Wachstein and Zak (1947), post-mortem material obtained more than 8 hours after death often shows an unreliable phosphatase pattern : it was therefore discarded. Quantitative analysis showed that the phosphatase remained for 48 hours after death, and material unsuitable for histology was sometimes used for chemical analysis. The hepatic sample was immediately divided into two portions One piece was used for chemical analysis, the other fixed immediately in absolute alcohol for histology.

The histochemical method was essentially that of Gomori. The tissue was incubated with buffered sodium glycerophosphate at a suitable *p*H, the phosphate split off and the ions trapped by calcium nitrate, forming the insoluble calcium phosphate The calcium phosphate was changed to cobalt phosphate,

which, acted upon by ammonium sulphide, is deposited as black cobalt sulp?. Various incubation times were tested. Two hours proved the most satisfac? in that it demonstrated little activity in sections from normal subjects compa with those seen in pathological states. In every instance a control section used in which the glycerophosphate was omitted. The counter-stain add by Kabat and Furth (1941) was found unnecessary and confusing. To ob comparative results a section from a preceding batch was included in each group of sections stained. The preparations were permanent.

Microchemical method. A portion of each liver specimen was wei? transferred to a small mortar and ground up with sand and a few drop 0·001 M phosphate buffer at pH 7·0. The mixture was carefully washed more buffer into a graduated cylinder and a 1 : 100 extract made. A few d of toluene were added and the extract left to autolyse at room tempera for 48 hours. The extract was then centrifuged and 0·2 ml. of the superna used for the test and 0·2 ml. for the control. The estimation was carried exactly as for phosphatase in serum (King and Armstrong, 1934), except the time of incubation was 30 minutes. If there was very little colour pro after 30 minutes' incubation, the estimation was repeated, incubating sample for 60 minutes and allowing for this in the calculation. Serum alka phosphatase was determined by the method of King and Armstrong. normal range is 5-10 units/100 ml. Serum bilirubin was determined by method of Haslewood and King (1937).

CLINICAL MATERIAL

1. *Normal subjects* (27). These were patients convalescent from non-he diseases. In every instance in which they were investigated, the serum-bilirub? concentration was less than 1·0 mg/100 ml. and the serum alkaline phosphatase less than 10 units/100 ml.

2. *Acute hepatitis* (28 cases). This group included simple infectious hepatitis (15 cases), jaundice following serum infusions (2 cases) and jaundice occurring during the course of arsenical therapy for syphilis (11 cases). The material was grouped according to the probable percentage of surviving hepatic cells. The grading was done on numbered slides from A to D, grade D being the most severe (Sherlock, 1946) : " diffuse " and " zonal " types of hepatitis were recognised (Dible, McMichael and Sherlock, 1943). In three patients the illness was of longer duration and the hepatic histology was that of subacute necrosis.

In 11 patients more than one biopsy was performed during the course of the illness. A further group of 8 had suffered from acute hepatitis two months to six years previously and had now made a clinical recovery.

3. *Obstructive jaundice* (26 cases). In every instance there was complete obstruction to the common bile duct, confirmed at operation or autopsy. Six suffered from gallstones, one from chronic pancreatitis, 3 from Hodgkin's disease or carcinoma of the stomach with enlarged glands in the porta hepatis, 11 from carcinoma of the ampulla of Vater or head of the pancreas and 5 from carcinoma of the main bile ducts.

In 9 instances more than one hepatic sample was available during the progress of the obstruction or after its release.

4. *Cirrhosis of the liver* (8 cases). An arbitrary division into active (4 cases) and latent (4 cases) was made.

5. *Generalised bone disease* (6 cases). In every instance there was radio logical or post-mortem evidence of bone involvement. The serum alkaline phosphatase was raised. Four suffered from Paget's disease of bone, one from bronchial carcinoma of the prostate with skeletal metastases and one from bronchial carcinoma with secondary deposits in bone.

RESULTS

The general histological distribution of the phosphatase is shown in table I. The results of chemical analysis and the serum enzyme levels are shown in tables II and III and fig. 1.

TABLE I

General histological distribution of alkaline phosphatase in the liver

	Liver cells	Hepatic sinusoids	Intralobular bile canaliculi
Normal subjects . . .	Mainly nuclear	Absent or central area only	Absent
Acute hepatitis .	Increased	Increased	Usually absent
Obstructive jaundice . .	Increased	Increased	Increased
Generalised bone disease .	Increased	Increased	Increased

TABLE II

Microchemical hepatic alkaline phosphatase

	No. of cases	Hepatic alkaline phosphatase (units/g.)		Serum alkaline phosphatase (units/100 ml.)		Serum bilirubin (mg./100 ml.)	
		Mean	Range	Mean	Range	Mean	Range
Normal .	17	2·40	5 6 0 4	6	9 3	0 6	1 0 0 5
Acute hepatitis .	11	6 78	18 0 1 0	21	48 12	11·2	22 0 3 6
Grade A .	3	2 14	3 8 1 0	16	22 12	6 0	10 0 3 6
Grade B .	3	3 26	4 2 2 6	21	30 14	11 5	11 0 5 0
Grade C . .	1	9 00		12	...	12 5	
Grade D .	2	13 60		17	21-12	15 0	18 0-12 0
Subacute necrosis .	2	11·10	13 8 8 4	35	48 23	17 5	21 0-14 0
Obstructive jaundice .	16	10 24	21 8 3 8	54	130 21	11 1	19 0 4 0
Generalised bone disease .	5	9 96	19 8 3 6	64	85 48	0 7	1 0 0 5

Normal subjects

Histology

Liver cells in every section show phosphatase activity in both nuclei and cytoplasm (figs 2 and 3). In the nucleus the enzyme is present in the nuclear membrane, chromatin network and nucleoli. The cytoplasm gives a faint brown reaction, with phosphatase-containing granules of various sizes inconstantly present in the cell. The enzyme is zonally distributed in the lobule. The cells adjoining the central vein show most, followed by the periportal area: the mid-zone shows least.

Sinusoids. The walls of the central hepatic veins constantly show enzyme activity (fig. 3). The walls of the sinusoids are variably stained. In 14 cases they are unstained. In 13 the sinusoid stains only as it

nuclear phosphatase only : the lining is not stained. Polymorph.
show their usual nuclear activity. Lymphocytes contain variable
amounts of phosphatase. Connective tissue does not give a reaction.

Microchemical values

The mean phosphatase value for 17 subjects was 2·4 (range 5·6·0·4)
units/g. In only five instances was the hepatic phosphatase level
greater than 4 units/g. (fig. 1).

Serum phosphatase values were always within normal limits (fig. 1).

Acute hepatitis

Histology of acute stage

Liver cells contain variable amounts of phosphatase. Surviving
cells usually show an increase in both cytoplasm and nucleus. The
general colour of the cell is darker and the number and size of the
granules excessive. Maximal cell damage is in the centres of the
lobules. Here many of the necrotic cells contain quantities of bile
pigment. In Gomori-stained preparations these cells show large
quantities of enzyme (fig. 7), cytoplasmic details being obscured.

Sinusoids. The wall of the central vein is constantly stained.
The walls of the sinusoids show greater enzymatic activity and are
much thicker than normal (figs. 4 and 7). The sinusoids are stained
in every section and in 18 out of 28 cases this extends throughout the
lobule, always being maximal centrally. The disappearance of central
cells has led to condensation of the sinusoids (fig. 7). The proliferated
sinusoidal cells and the infiltrating polymorphs and lymphocytes
show great nuclear and to a less extent cytoplasmic activity.

Intralobular bile canaliculi are again inconspicuous. In 6 only of
the 28 cases is there patchy filling with phosphatase. The canaliculi
are thin. Central zone bile thrombi show much phosphatase. Some-
times the thrombus is more darkly stained at its centre than at the
periphery (fig. 8).

Portal tracts. The portal vessels show their usual phosphatase
activity. The proliferated bile ducts show nuclear phosphatase but
none in the cytoplasm of their lining epithelium. The conspicuous
portal cellular infiltration usually gives a strong reaction (fig. 5),
the nuclei of polymorphs, lymphocytes and histiocytes being black.
Although the older connective tissue shows only nuclear staining, the
occasional young fibroblasts have both cytoplasmic and nuclear
phosphatase.

**Relation of phosphatase activity to severity and type of
hepatitis.** In general, the more widespread the necrosis, the more
phosphatase is histologically demonstrable in the liver. The necrotic
areas consist of disintegrating liver cells, collapsed and condensed

FIG. 2.—Convalescent duodenal ulcer, J. B., aged 54. Liver showing normal phosphatase activity. Serum phosphatase 5 units/100 ml.; hepatic phosphatase 1·0 units/g. × 145.

FIG. 3.—Same case as in fig. 2. Centre of lobule showing phosphatase in the walls of the central vein and sinusoids. Phosphatase is also seen in the hepatic cell nuclei and cytoplasm. × 335.
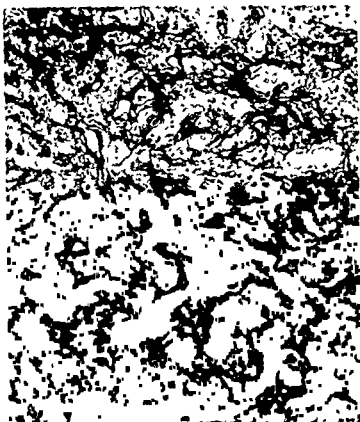
FIG. 4.—Acute hepatitis, case 1, F. M., aged 60, jaundiced 9 days. Serum bilirubin 14·6 mg./100 ml., phosphatase 17 units/100 ml. Grade D hepatitis. Sinusoids contain much phosphatase. Many liver cells have disappeared, surviving cells contain a normal amount of phosphatase. Bile canaliculi are not stained. The portal tracts show phosphatase only in the walls of the blood vessels. × 120.

sinusoids, lymphocytes and red cells, all of which show much enzyme (fig. 4). The diffuse type of hepatitis is therefore associated with maximal phosphatase increase. The phosphatase content of the zonal type is variable, largely depending on how much is present in the portal tract cellular accumulations. This is unpredictable from case to case, or from time to time in any one case (*cf.* figs. 4 and 5).

Subacute hepatic necrosis. All three cases show remarkable increases in phosphatase. Surviving parenchymal nodules contain either a normal or an excessive amount, both cytoplasmic and nuclear. Cells isolated in areas of necrosis show very variable amounts. Some contain none; others, which in control sections show bile, have excessive amounts. Occasionally the remaining liver cells assume an acinar appearance and surround plugs of bile which show phosphatase activity (fig. 8). The wide areas of necrosis between the liver cell nodules, as in the acute stage of hepatitis, give a strong phosphatase reaction (fig. 8).

Changes during recovery from acute hepatitis. The speed of liver cell recovery is very rapid. The excessive hepatic phosphatase decreases much more slowly. With recovery the dilatation of the central sinusoids is no longer seen. The necrotic cells are removed and replaced by liver cells with a normal phosphatase content. The enzyme in the sinusoidal walls persists and is often increased (*cf.* figs. 4 and 5), and may spread further through the lobule. Occasionally for the first time phosphatase appears in the bile canaliculi. The portal tracts with their lymphocytes and young fibroblasts usually show much phosphatase (fig. 6).

Return to the normal distribution occurs a variable time after the beginning of recovery. Multiple biopsies were performed in 11 patients. In some, routine sections show healing apart from portal tract scars: in others, studied earlier, the cellular recovery is not so complete. In 7 patients 9-66 days (mean 33 days) after the first biopsy there is no diminution. In another three an interval of 25-35 days resulted in some decrease, but the amount of phosphatase was still greater than normal. This also applied in the case illustrated 51 days after the first biopsy (figs. 4-6).

In a further group of 8 cases, the liver biopsy was performed for the first time when clinical recovery from acute hepatitis was complete. Routine sections showed normal hepatic histology, apart from some portal tract scarring. In three cases 6 years, 19 months and 7 months after the acute attack the distribution of hepatic alkaline phosphatase was normal. In the other five, studied 2-9 months after the acute attack (mean 5·4 months), there was a conspicuous increase in the phosphatase, mainly in the sinusoidal walls.

Microchemical values

Results were scattered, but there was a significant increase of the mean over that for normal subjects (table II and fig. 1). In general, the more severe the liver cell damage, the higher was the concentration of hepatic phosphatase (table II). The truth of this observation cannot be established on such a small series. If, however, the values for the milder grades A and B are compared with those of the more severe grades C and D and for subacute hepatic necrosis, a conspicuous increase in the latter is seen. Furthermore, the value for each patient in grades C and D and in subacute necrosis is greater than that for each patient in grades A and B. In two patients the hepatic alkaline phosphatase was measured during the acute stage and after clinical recovery. In neither was there a significant fall (table III). In two other subjects a raised hepatic phosphatase—8·8 and 6·6 units/g.— was obtained 9 and 6 months after clinical recovery from the hepatitis.

Serum phosphatase values. In 26 of the 28 subjects the serum phosphatase was greater than 10 units/100 ml. There was little correlation between the serum and hepatic phosphatase (fig. 1). During recovery, the slow fall in hepatic phosphatase was associated with a slow fall in the serum level. In 7 of the 11 subjects in whom a second biopsy was performed, although the serum bilirubin concentration was less than 2 mg./100 ml., the serum phosphatase was still greater than 10 units/100 ml. Of the five cases studied at a later period (2-9 months after recovery), only one had a serum phosphatase greater than 10 units/100 ml. This suggests that the serum alkaline phosphatase, although slow to reach normal, does so more quickly than the hepatic value.

Illustrative case 1

F. M., 59-year-old conductor. 10.2.44 ; had his last arsenic injection for neuro-syphilis. 26.2.44 ; anorexia and epigastric pain. 28.2.44 ; jaundiced. 8.3.44 ; admitted to hospital deeply jaundiced. Liver enlarged 6 cm. below right costal margin, smooth and tender. Spleen also palpable. Bile pigment present in urine but no urobilin. Clinical diagnosis :—arsenotherapy "serum" jaundice. 9.3.44 ; serum bilirubin 14·6 mg./100 ml., alkaline·phosphatase 17 units/100 ml.

First aspiration biopsy (fig. 4) shows an extremely severe hepatitis (grade D). Phosphatase-stained sections show the disappearance of the liver cells, their place being partially taken by collapsed sinusoids showing great enzyme activity. Phosphatase is not seen in the canaliculi : portal tracts contain phosphatase only in the walls of the blood vessels.

18.3.44 ; drowsy ; rectal bleeding ; pitting sacral œdema. 21.3.44 ; feeling better ; urobilin present in the urine ; serum bilirubin 14 mg./100 ml., alkaline phosphatase 11·5 units/100 ml.

Second aspiration biopsy (fig. 5) shows the remarkable degree of cellular recovery. Sinusoidal walls still heavily stained ; patchy filling of bile canaliculi. Portal tracts now show phosphatase activity in lymphocytes and in new fibrous tissue.

3.4.44 ; recovering uneventfully ; serum bilirubin 2·6 mg./100 ml., phosphatase 15·5 units/100 ml.
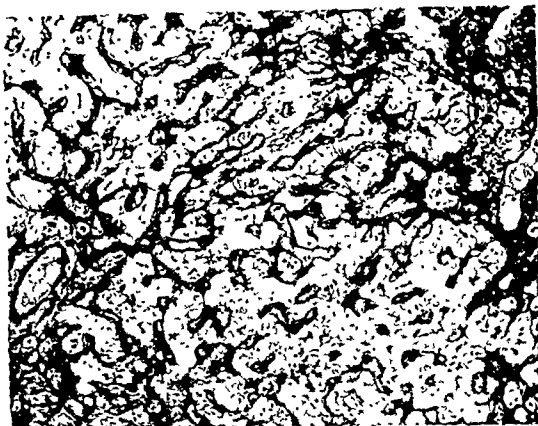
FIG 5 —The same case as in fig. 4, 21st day of jaundice   Serum bilirubin 14 mg /100 ml ,
phosphatase 21·5 units/100 ml   There has been great regeneration of liver cells.
Much phosphatase is seen in the walls of the sinusoids   There is patchy filling of the
bile canaliculi with phosphatase.   Portal tracts also show excess of enzyme   × 135.
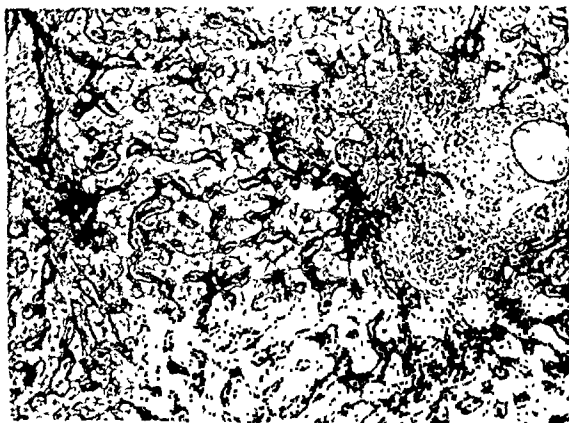
FIG 6 —Same case as in figs 4 and 5 , 72nd day of illness   Serum bilirubin 0 5 mg./100
ml , phosphatase 12 units/100 ml.   Liver cell restitution is almost complete   The
amount of phosphatase is slightly less than is shown in fig 5, but is still increased
in sinusoids and portal tracts   Scattered bile canaliculi also show phosphatase.
× 135.

HEPATIC ALKALINE PHOSPHATASE
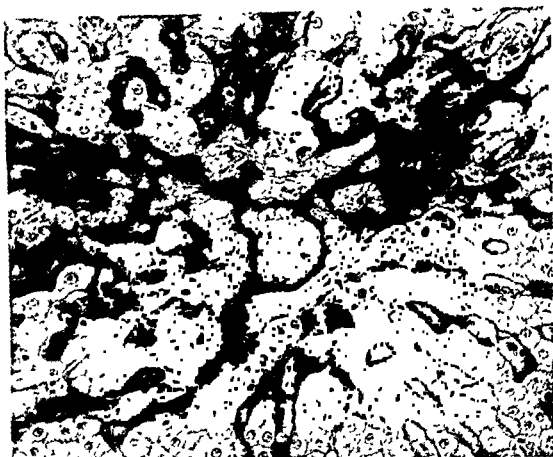
FIG. 7.—Acute hepatitis, F. P., aged 32; jaundiced 10 days. Serum bilirubin 18 mg./100 ml., phosphatase 12 units/100 ml. Centre of lobule showing intense phosphatase activity in the walls of the central vein and sinusoids. Some necrotic liver cells also show phosphatase. The bile canaliculi are not seen. ×235.
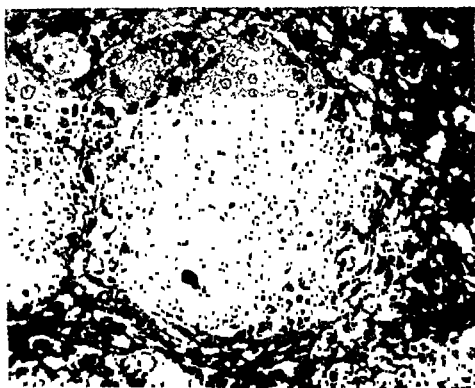
FIG 8 —Subacute hepatic necrosis, F., aged 3 months; 13 days jaundiced. Serum bilirubin 22 5 mg./100 ml., phosphatase 48 units/100 ml.; hepatic phosphatase 8 4 units/g The nodule of surviving liver cells contains normal amounts of phosphatase. Liver cells surround a bile thrombus which shows phosphatase activity. Surrounding collapsed sinusoids, remains of liver cells, lymphocytes and connective tissue show much phosphatase. ×240.

HEPATIC ALKALINE PHOSPHATASE
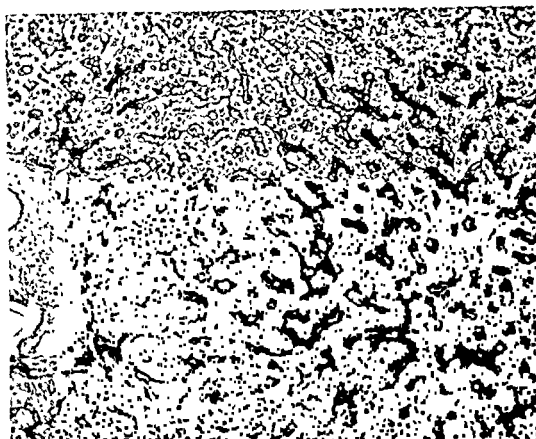
FIG. 9.—Obstructive jaundice, M. D., aged 27. Gall stones. Jaundiced 44 days. Serum bilirubin 6 6 mg./100 ml., phosphatase 31 units/100 ml. ; hepatic phosphatase 8 0 units/g. Phosphatase is greatly increased in liver cells, sinusoids and bile canaliculi. Bile thrombi at centre of lobule show activity. × 120.
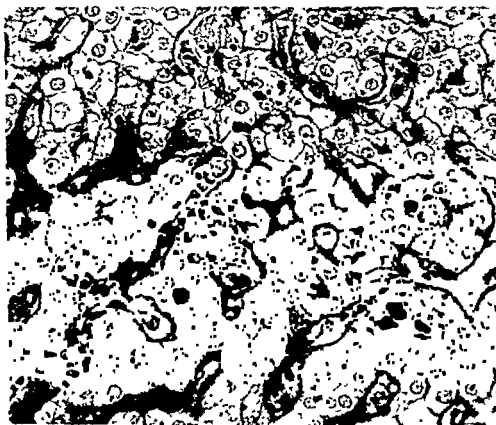
FIG. 10.—Obstructive jaundice. Phosphatase is seen in the sinusoids, in the dilated bile canaliculi and in bile thrombi There is also an increase in the liver-cell phosphatase. × 280.

HEPATIC ALKALINE PHOSPHATASE
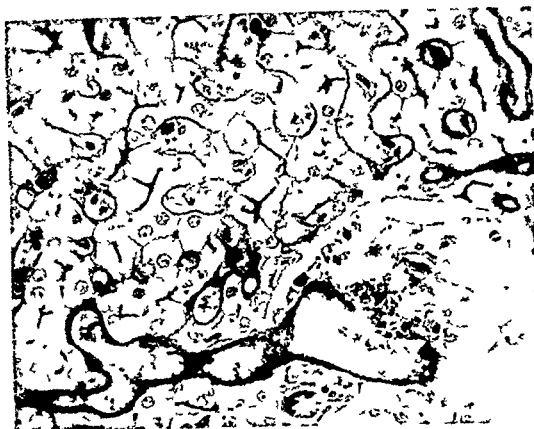
Fig 11—Bronchial carcinoma with skeletal metastases   Phosphatase is seen in the sinusoids and bile canaliculi   ×340
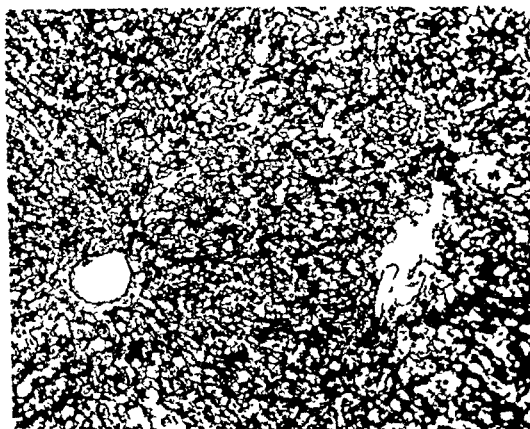
Fig 12—Paget's disease of bone L , aged 60   Serum phosphatase 50 units/100 ml hepatic phosphatase 19 8 units/g   A very intense phosphatase reaction is seen in the liver cells, sinusoids and bile canaliculi   ×135

Third aspiration biopsy shows further recovery of liver cells towards the central vein. Sinusoidal phosphatase is less, and the canalicular enzyme is still patchy. Portal tract infiltrations are still present.

2.5.44; complete clinical recovery, liver now just palpable; serum bilirubin 0·5 mg./100 ml.; alkaline phosphatase 12 units/100 ml.

Fourth aspiration biopsy (fig. 6). Excessive phosphatase still present, but less than in biopsy 3. Sinusoidal and canalicular phosphatase still obvious.

The points to be noted are :—(1) the increased hepatic phosphatase at the acute stage of this very severe hepatitis; (2) the slow disappearance of the enzyme from the liver, excess still being present on the 60th day of illness when clinical recovery was complete; (3) the appearance of phosphatase in the bile canaliculi during recovery.

Obstructive jaundice

Histology

Liver cells. There is a constant increase of phosphatase in the cytoplasm, both granular and diffuse. The granules, as usual, are mainly in the central cells and to a lesser extent in the periportal zone. Unstained control sections show that the increased granules often correspond with increased intracellular bile pigment. The nuclear chromatin membrane and nucleoli also show a heavy increase in phosphatase.

Sinusoids. The sinusoidal walls show a constantly increased activity over that seen in normal subjects (fig. 9). The most conspicuous increase is towards the central vein, although the sinusoidal walls are often active throughout the lobule, with a further increase at the periphery. There is sometimes distension of the sinusoids as they approach the central vein. Their walls are thickened, and sometimes they appear full of phosphatase (fig. 10). Sinusoidal endothelial cells, Kupffer cells and polymorphs show increased activity.

Bile canaliculi, thrombi and necroses. In every case there is conspicuous canalicular enzyme. In 11 instances this extends throughout the lobule : in the other 15 it is confined to the central and periportal regions. The distribution is never patchy, as described in acute hepatitis. The channels are widened and distended, both in their intra- and extra-cellular courses (fig. 10). The focal biliary necroses of obstructive jaundice consist of a central extravasation of bile surrounded by disintegrated liver cells. The bile shows phosphatase activity, often confined to its centre : the surrounding zone shows only nuclear enzyme.

Portal tracts. The bile duct epithelium shows no cytoplasmic phosphatase, though it does show intense nuclear enzyme. Sometimes the bile ducts contain plugs of bile, and these also show much phosphatase. There is staining of the polymorphs and of the endothelium of the portal vein and hepatic artery. Lymphocytes and fibroblasts stain variably.

Relation to duration of obstruction. On the basis of this small series a close relationship between the duration of the obstruc-

tion and the histological amount of phosphatase cannot be established. Of four patients in whom jaundice had existed for less than 14 days, three showed amounts below the average for the whole series, the fourth was above average. After about a fortnight there seemed to be little correlation with duration. This was confirmed when serial biopsies from five patients during the course of unrelieved obstruction were studied. One only increased from the 48th to the 100th day. Two others apparently diminished from the 63rd to the 76th and from the 28th to the 76th day. The remaining two, studied on the 37th and 81st days and on the 14th and 65th days, showed little change in the amount of phosphatase histologically demonstrable.

The effect of relief of obstruction. Excess phosphatase was slow to disappear from the liver. Sections before and after relief of obstruction were available in six patients. In three, 6-8 weeks after relief, the serum bilirubin was 2-3 mg. per 100 ml. and the hepatic phosphatase picture is unchanged. In three, 2-6 months after operation, the serum bilirubin was 0·5-1·4 mg. per 100 ml. and increased cellular (sinusoidal and canalicular) phosphatase is still present, but in diminished amount. In all six cases bile thrombi showing phosphatase activity are still present.

Microchemical values

Scatter is again large, but there is a very significant increase in the mean over that for normal subjects (fig. 1 and table II).

A patient in whom jaundice had been noted for only 5 days had the lowest value (3·8 units/g.). The other estimations were done on samples from patients jaundiced more than 14 days. In these there was no correlation between the duration of the jaundice and the quantity of phosphatase in the liver (table III).

Hepatic phosphatase diminished very slowly after surgical or spontaneous relief of obstruction. Of the four cases in table III, 41-195 days after relief the serum bilirubin concentration was less than 2 mg./100 ml. but there was still a conspicuous increase in the hepatic phosphatase.

Serum phosphatase values. In general, the higher serum levels were associated with higher hepatic values (fig. 1). Serum phosphatase usually increases for the first 3 weeks of jaundice. Values then fluctuate, the trend being upward. This corresponds with the histochemical and microchemical changes in hepatic phosphatase.

Illustrative case 2

F. B., housewife, aged 70 (case 1, table III). 4 weeks' progressive jaundice following severe abdominal pain and vomiting. No loss of weight. On examination, deeply jaundiced, liver enlarged 4 cm., gall-bladder not palpable; urine contained bile but no urobilin. Clinical diagnosis:—stone in common bile duct.

Aspiration biopsy 1, 2 6 46   Sections show features of obstructive jaundice. Hepatic cell, sinusoidal and canalicular phosphatase is excessive. Bile thrombi show enzyme activity.

10 6 46   patient recovered spontaneously.

Aspiration biopsy 2.   Sections show a diminution in the amount of enzyme, which, however, is greater than normal. The canaliculi are still filled with phosphatase.

The changes in serum bilirubin and phosphatase concentration and in the hepatic phosphatase are shown in table III. Serum bilirubin fell. Serum phosphatase was halved, with approximate halving of the hepatic phosphatase. Patient left hospital 5 8 46. Symptoms, including jaundice, recurred, and on 29 10 46 the patient was admitted. Cholecystectomy was performed 11.11.46; the gall bladder and common bile duct were full of stones.

Operation hepatic biopsy showed a histological picture very similar to aspiration biopsy 1. Although jaundice was not so deep the serum and hepatic phosphatase concentrations were as high as on the first admission.

Post operative recovery was uneventful. Unfortunately the patient was re admitted with symptoms of intestinal obstruction and died on 21.2 47 before a further operation could be undertaken. Post mortem examination showed small intestinal obstruction from post operative adhesions

Post mortem liver sections show less phosphatase in the liver than on 11.11.46, but sinusoidal and canalicular increase over normal is still seen. Serum and hepatic phosphatase had quantitatively diminished, but were still greater than normal   Serum bilirubin was normal.

Points to be noted are (1) the close correlation between histological appearances and microchemical and serum phosphatase values during the course of the illness ; (2) the failure of serum and hepatic phosphatase to reach normal 102 days after final relief of the biliary obstruction.

Hepatic cirrhosis

Histology

Inactive.   Hepatic phosphatase is not increased ; liver cell nuclei and cytoplasm show their usual complement and there is none in sinusoids or canaliculi. The interlacing bands of fibrous tissue are mature and contain only a little nuclear enzyme   Portal vein and hepatic artery branches and the nuclei of bile ducts give their normal reaction

Active   There is a change in the distribution. Cytoplasmic phosphatase granules are variable, sometimes increased, sometimes diminished   Cells distended with fat contain normal amounts, the phosphatase being pushed to the side of the cell with the nucleus.

The hyperplastic nodules show activity in their sinusoidal walls, and at the periphery the sinusoids are condensed by the disappearance of liver cells (fig. 15). Occasionally bile channels are seen forming a thin outline around the liver cells ; often they are not obvious. The blood vessels and the nuclei of the bile-ducts in the connective tissue bands between the pseudo-lobules show activity. Polymorphs contain much phosphatase. Young fibroblasts show both cytoplasmic and nuclear enzyme. The more mature fibrous tissue gives only nuclear reaction.

Serum phosphatase values

These were normal in the latent and moderately increased (10-25 units/100 ml.) in the active group.

Generalised bone disease with raised serum alkaline phosphatase

Histology

Routine histology in every case was normal.

Liver cells. Five of the six cases show increase of both nuclear and cytoplasmic enzyme. This is most conspicuous at the centres of lobules.

Sinusoids. In every instance the sinusoidal phosphatase is increased. In the least severe it extends only from the central vein half-way through the lobule; in the most severe every sinusoid is demarcated (fig. 12).

Bile canaliculi. In five of the six cases phosphatase is present in the canaliculi. In two it merely outlines the liver cells: in the other three there is very deep staining of the intra- and extra-cellular canaliculi throughout the section, being maximal towards the central vein and periportal vein. The canaliculi are never as thick or as distended as in obstructive jaundice (*cf.* figs. 10 and 11). Bile thrombi are not seen.

Microchemical values

In four of the five patients the hepatic phosphatase was greater than 7·0 units/g. (table II). In the fifth case the value (3·6 units/g.) was within the normal range but higher than the normal mean.

Serum phosphatase values. These were raised in all six cases. There seemed to be little relationship between hepatic and serum concentrations (fig. 1).

Illustrative case 3

A. V., clerk, aged 60. Admitted to hospital 14.1.47 with shooting pains in right buttock, thigh and foot. In 1939 he had a one-stage prostatectomy. On examination an ill man; liver not palpable; tender over second sacral vertebra; bilateral leg wasting with absent ankle reflexes. X-ray appearances of pelvis suggest secondary deposits from carcinoma of the prostate.

20.2.47; serum alkaline phosphatase 85·5 units/100 ml., serum acid phosphatase 16·5 units/100 ml., of which 10·5 units were formol-resistant (Abul-Fadl and King, 1947).

Aspiration biopsy 1 (fig. 13). There is a great increase in hepatic phosphatase in the lining cells of the sinusoids and canaliculi. The quantitative hepatic phosphatase was 7·2 units/g. The patient was then given a daily intramuscular dose of 40 mg. of œstradiol benzoate.

1.5.47; symptoms had diminished. There was no radiological change in the bones. Serum alkaline phosphatase 40 units/100 ml., serum acid phosphatase 21·5 units/100 ml., of which 5 units were formol-resistant.

Aspiration biopsy 2 (fig. 14). There is a diminution in liver-cell, sinusoidal and canalicular phosphatase. Quantitative hepatic phosphatase 4·6 units/g.

The points to be noted are (1) the distribution of the enzyme in liver cells, sinusoids and canaliculi; (2) the fall in serum and hepatic phosphatase following œstrogen therapy.

Fig. 13 —Prostatic carcinoma with skeletal metastases, case 3, A. V., aged 60. Serum alkaline phosphatase 83 5 units/100 ml , hepatic phosphatase 7 2 units/g. Phosphatase is seen in liver cells, sinusoids and canaliculi × 125

Fig. 14.—Same case as in fig. 13, 44 days later, after œstrogen treatment. Serum alkaline phosphatase 40 units/100 ml , hepatic phosphatase 4·6 units/g. The hepatic phosphatase has diminished It is seen in the sinusoids and in the bile canaliculi at the periphery of the lobule. × 125.
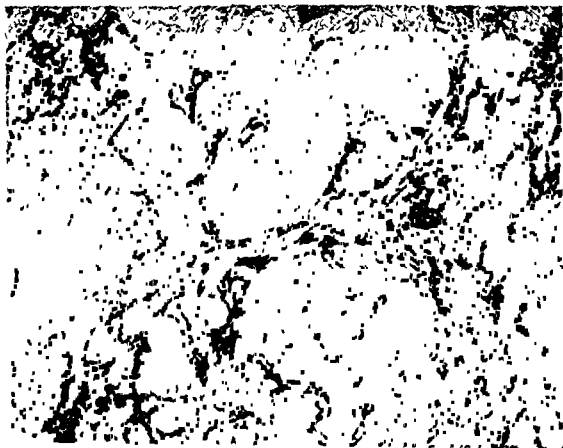
Fig 15.—Hepatic cirrhosis, S H., aged 35. Serum bilirubin 1 3 mg /100 ml , phosphatase 14 units/100 ml Phosphatase is seen in the walls of the sinusoids and in the bands of connective tissue. Proliferating bile ducts show only nuclear phosphatase. × 145.

DISCUSSION

One of the purposes of this investigation was to decide whether Gomori's histological method was a true reflection of quantitative changes in the hepatic alkaline phosphatase. It was also hoped to obtain values for hepatic phosphatase that might be subjected to statistical analysis. It was gratifying to discover a fairly good agreement of the results obtained by histological and chemical methods. That the correlation is not closer is not surprising. Indeed, study of sections stained for phosphatase did not enable a prediction of the exact results of chemical analysis to be made with any certainty, though a rough quantitative assessment was usually possible.

There are various difficulties in making a comparison of the two methods. There must be a small sampling error even though adjacent areas were taken for the two methods. On the other hand, analysis of multiple larger samples obtained at operation has shown good agreement between neighbouring portions of liver. This sampling error will obviously be less in normal subjects and in obstructive jaundice, where the histological picture is reasonably uniform, than in acute hepatitis, where the pathological process is more patchy. In cirrhosis the liver lesion is so irregular that no attempt at chemical analysis was made.

Another source of error in the small biopsy samples is the irregular distribution of alkaline phosphatase in the lobule itself. There is a variation from one biopsy to another in the number of portal tracts or central veins and hence analytical values may also differ. This error is lessened as the number of samples is increased.

Another difficulty lies in comparing results from alcohol-fixed and fresh tissue. The alcohol dissolves out blood from the sinusoids, but chemical analysis includes this. In patients with similar serum phosphatase values this error will be about the same : where these differ, the difficulty is unavoidable. The poor correlation between hepatic and serum phosphatase levels suggest that it is unimportant.

Although a hepatic origin for serum phosphatase has been suggested (Bodansky and Jaffe, 1933-34; Greene *et al.*, 1934; Freeman *et al.*, 1938), other workers believe that the liver merely excretes it (Armstrong, King and Harris, 1934; Armstrong and King, 1935). Our findings are in keeping with the latter view.

If the liver were the main source of alkaline phosphatase the hepatic level would be expected to fall rather than rise with increasing severity of hepatitis. Moreover the enzyme in hepatitis is distributed in the sinusoids and liver cells and not in the bile channels. This suggests retention in both liver and blood, the injured liver cells having failed to excrete the phosphatase. In a few instances patchy filling of the canaliculi is seen. In these cases the small amount of phosphatase excreted by the liver cells is held up by the intralobular block of the bile channels. This intrahepatic obstruction in hepatitis

has already been described (Dible *et al.*). With recovery the block is relieved, with resulting flooding of the bile canaliculi with phosphatase.

In obstructive jaundice phosphatase is seen wherever there is bile stagnation. It is therefore demonstrated in the bile-stained liver cells and in the bile canaliculi, in bile thrombi and bile necroses. The excretion of the phosphatase in the bile is clearly being prevented by the biliary obstruction. The enzyme in the walls of the hepatic sinusoids could be a reflection of an increased serum level or could represent obstruction to the excretion of phosphatase by the liver. The obstructive jaundice studies therefore give little information as to the source of hepatic alkaline phosphatase.

In generalised bone disease, routine histological staining has failed to demonstrate a hepatic lesion, yet the hepatic phosphatase is quantitatively increased and the histological distribution is identical with that of obstructive jaundice. In these cases the bone disease is associated with a rise in serum phosphatase and an increase not only in the sinusoids but also in the bile canaliculi. Moreover, treatment of the bone condition with consequent fall in serum phosphatase is associated with a quantitative diminution in hepatic phosphatase in both sinusoids and bile canaliculi.

Both Wachstein and Zak (1946, 1947), studying Gomori-stained sections of liver from the dog and also human post-mortem material, and Jacoby (1947), using rat's liver, have found a remarkable increase in bile capillary phosphatase in obstructive jaundice. Wachstein and Zak (1946) conclude that the increase in serum phosphatase in liver damage is due to inability of the liver cells to excrete the enzyme rather than to increase production in the liver itself. Biopsy material and microchemical analysis have not hitherto been used for phosphatase studies in man. Further investigations along these lines are needed. Concomitant estimation of the bile phosphatase level in experimental bile fistulæ, especially in generalised bone disease, may provide further useful information.

Morris and Peden (1937) suggest that the increased serum phosphatase in obstructive jaundice can be related to the absence of bile in the intestine. This is said to interfere with fat and calcium absorption and to result in osteoporosis. Phosphatase is released from the bone in large quantities. The increased serum and hepatic phosphatase, not only in jaundice but also in bone disease, might therefore have a similar causation, namely increased production by bone. Various observations make this theory unlikely. Relief of an obstructive jaundice by an external biliary fistula results in a fall in serum phosphatase, even though bile has not reached the intestine. Return of fistula bile to the intestine does not alter the rate of fall of the serum phosphatase. The serum phosphatase in obstructive jaundice cannot be reduced by the intravenous injection of calcium (Sherlock, unpublished observations). Moreover, in dogs, ligation of

one hepatic duct causes a rise in the serum-phosphatase level (Schiffmann and Winkelman, 1939).

The explanation of the slow rate of disappearance of phosphatase from the liver during recovery from acute hepatitis and obstructive jaundice is uncertain. The liver seems to find the excretion of phosphatase more difficult than that of bilirubin, thus providing an interesting correlation with the slow fall of the serum phosphatase already noted during recovery from hepatitis (Sherlock, 1946).

SUMMARY

1. The histological distribution and microchemical analysis of alkaline phosphatase in the liver in normal subjects, acute hepatitis, obstructive jaundice, hepatic cirrhosis and generalised bone disease have been studied, using material obtained by aspiration biopsy, at operation, and in a few instances, at autopsy. Results have been compared with the serum phosphatase levels.

2. In normal subjects the phosphatase is found in the hepatic cell nuclei. Minimal amounts only are present in the sinusoidal walls. The mean hepatic alkaline phosphatase is 2·40 units/g., the mean serum enzyme 6·2 King-Armstrong units/100 ml.

3. In acute hepatitis the phosphatase is increased in both hepatic cells and nuclei. The sinusoidal walls contain large amounts. The bile canaliculi do not usually contain the enzyme. The mean hepatic alkaline phosphatase is 6·78 units/g. The mean serum enzyme value is 21 units/100 ml. Histologically and chemically the hepatic phosphatase increases with increasing severity of the hepatitis. Recovery results in very slow diminution of the hepatic enzyme.

4. In obstructive jaundice the phosphatase is increased both in the hepatic cells and in the walls of the sinusoids. The bile canaliculi are full of the enzyme. The mean hepatic alkaline phosphatase is 10·24 units/g., the mean serum enzyme 54 units/100 ml. After the first fortnight of jaundice there is little correlation with the duration of the jaundice. Relief of obstruction results in very slow diminution of hepatic phosphatase.

5. In inactive hepatic cirrhosis hepatic phosphatase is not increased. Serum values are normal. In active cirrhosis the enzyme is increased in the sinusoids of the pseudo-lobules of liver cells and in the connective tissue bands. Serum alkaline phosphatase is increased to 10-25 units/100 ml.

6. In generalised bone disease with a mean serum phosphatase of 64 units/100 ml. there is a great increase in hepatic alkaline phosphatase in the cells, sinusoidal walls and bile canaliculi. The mean hepatic alkaline phosphatase is 9·96 units/g. Œstrogen treatment of a case of prostatic carcinoma with skeletal metastases resulted in a fall of both serum and hepatic alkaline phosphatase.

7. There is fairly good agreement between the histological and

has already been described (Dible *et al.*). With recovery the block is relieved, with resulting flooding of the bile canaliculi with phosphatase.

In obstructive jaundice phosphatase is seen wherever there is bile stagnation. It is therefore demonstrated in the bile-stained liver cells and in the bile canaliculi, in bile thrombi and bile necroses. The excretion of the phosphatase in the bile is clearly being prevented by the biliary obstruction. The enzyme in the walls of the hepatic sinusoids could be a reflection of an increased serum level or could represent obstruction to the excretion of phosphatase by the liver. The obstructive jaundice studies therefore give little information as to the source of hepatic alkaline phosphatase.

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OBSERVATIONS ON THE PREVENTION OF BAC-TERIAL GROWTH BY SULPHONAMIDES, WITH SPECIAL REFERENCE TO THE HARPER AND CAWSTON EFFECT

N. WALKER, R. P. PHILIP, M. W. SMYTH and J. W. McLEOD

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(PLATE XCVII)

Corrigendum

The correct names of the authors of the paper commencing on p. 631 are:—

N. WALKER, R. PHILIP, M. M. SMYTH and J. W. McLEOD

However, during a comparison of penicillin and other antiseptic substances in cup plates, the rather surprising fact was noted that in horse-blood agar a marked zone of inhibition of staphylococcal growth could be obtained in the vicinity of cups which contained relatively high concentrations of sulphonamide solutions (0·5-1 per cent.), although similar results were not obtained with plain agar nor to any considerable extent when other types of blood were added to the medium. This phenomenon was extensively investigated on the assumption that the horse blood exerted a peculiar potentiating effect on the sulphonamide.

While this work was still in progress, Harper and Cawston (1945) published a paper in which attention was drawn to the same phenomenon. The interpretation which these authors put on their observations was that the horse blood destroyed the substances in nutrient broth which acted as inhibitors of sulphonamide activity. They were unable to obtain evidence, however, that the effect of p-aminobenzoic acid was diminished under these conditions and hence assumed the activity of sulphonamide inhibitors other than p-aminobenzoic acid.

Finally on the basis of these observations they recommended the preparation of a medium specially suitable for determining the activity of sulphonamides against bacteria. This was obtained by incubating broth with horse blood overnight, steaming the resulting fluid for 30 minutes, filtering off the blood and protein coagulum and then incorporating agar in the filtrate.

Observations confirmatory of Harper and Cawston's results

The confirmation of Harper and Cawston's principal observation was readily obtained. Medium was prepared from a mixture of equal parts of non-nutrient agar and a filtrate of a broth containing 10 per cent. of hæmolysed horse blood which had been incubated
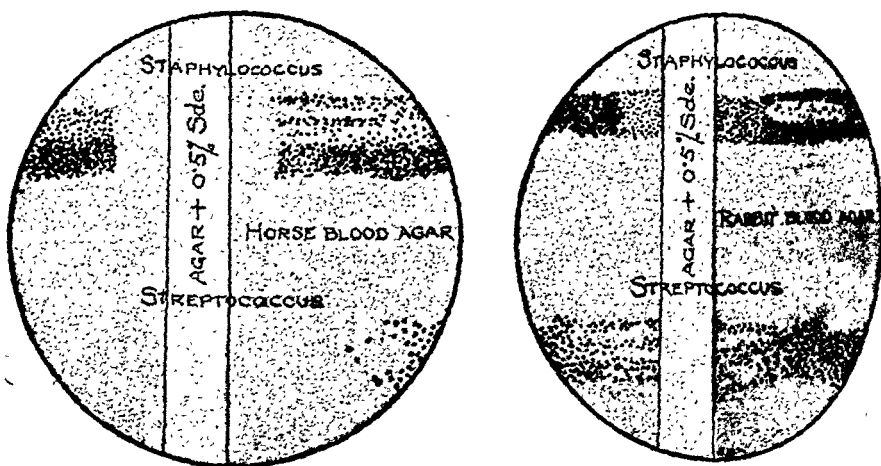
FIG. 1.—Comparative effects of horse and rabbit blood on the inhibition of staphylococci and streptococci by sulphonamide.

overnight and steamed for 30 minutes. When a trench of non-nutrient agar containing 0·5–1 per cent. of sulphonamide was introduced into this agar, cross inoculation with three dilutions of a culture of staphylococcus showed complete or almost complete inhibition of staphylococcal growth to a distance of $\frac{1}{4}$–$\frac{3}{4}$ in. from the trench. The effect varied a little with the weight of inoculum of staphylococcus and the particular sample of nutrient broth; it was little if at all inferior to that obtained when the hæmolysed horse blood was incorporated with the nutrient agar forthwith and the mixture poured as a plate without heating. Fig. 1 shows the contrast between horse- and rabbit-blood agar plates in such observations. Agar prepared by Harper and Cawston's method gave results approximating to those shown for horse-blood nutrient agar.

It is possible to interpret this result in three ways : (1) Substances inhibitory to sulphonamide action may have been destroyed in the broth or removed from it ; (2) the action of horse blood on broth

may be to produce some system capable of potentiating the activity of sulphonamide ; or (3) both may happen simultaneously.

In view of the peculiar interest of this phenomenon—the change of an organism relatively insensitive to sulphonamide action both in bacteriological media and in the animal body to one highly sensitive in bacteriological media—it seemed well worth while to pursue the investigation in several directions.

The questions we have endeavoured to answer, which are unexplored or only partially explored in Harper and Cawston's work, are the following. (1) What is the element in horse blood responsible for this effect ? (2) Is the action of horse blood dependent on other substances present in the medium apart from the hypothetical inhibitory substances ? (3) What, if they exist, is the nature of the substances destroyed in nutrient broth ? (4) To what extent does the presence of horse blood promote the sulphonamide effect against bacteria other than the staphylococcus ? (5) Can any system potentiating the sulphonamide effect be demonstrated in media treated with horse blood ?

THE ELEMENT IN HORSE BLOOD RESPONSIBLE FOR PROMOTING SULPHONAMIDE ACTION

The first point noted was that horse blood was markedly superior to most others. Harper and Cawston's observations were similar. In addition to the bloods of the species which they tested—horse, human, rabbit and sheep—we also examined ox, dog, cat, guinea-pig, monkey, pig, rat, hen, pigeon and frog bloods, and found them relatively inactive.

Asses' blood, however, was found to be little if at all inferior to horse blood and the property would therefore appear to be one peculiar to the equine species. We have been unable to obtain samples of zebra or giraffe blood.

In attempting to locate the effect in some part of the blood we have examined the following : heated blood, unhæmolysed blood, hæmolysed blood (equal parts H_2O and blood), blood rich in leucocytes obtained by taking the upper layers of centrifuged blood, filtrate of hæmolysed blood and a solution of separated horse-hæmoglobin crystals.

The effects were better with hæmolysed than with unhæmolysed blood and the presence of excess of leucocytes did not increase the action. The activity of hæmolysed blood was unimpaired after Maassen filtration ; hence the stromata of the corpuscles were not involved. If the blood-broth mixture or blood-agar mixture was heated at once, much less activation of sulphonamide was obtained in the mixture. To our surprise, however, a solution of crystalline hæmoglobin was found relatively ineffective. The method of Keilin and Hartree (1935) was followed in preparing crystalline hæmoglobin.

It was observed that the effect was little if at all impaired if the crystals separated at the first stage were used. It was largely or entirely eliminated, however, if the crystals were taken which separated in the second stage, *i.e.* after solution of the first crop of crystals in N NaOH and neutralisation with N HCl. There are therefore grounds for assuming that the effect was related to the hæmoglobin or some very closely associated substance which could be inactivated by heating for 15 minutes at 70-75° C.

This conclusion was further borne out by observations on the inhibition of growth in plates containing trenches of hæmolysed blood agar introduced into a medium of nutrient agar containing $\frac{1}{2}$-1 per cent. of sulphanilamide. In such plates growth occurred
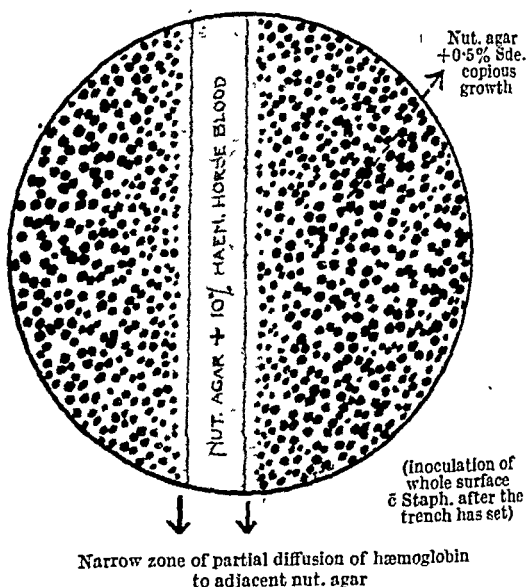
FIG. 2.—Inhibition of staphylococci by sulphonamide is limited to the zone into which hæmoglobin has diffused.

over the whole surface except the trench and a very narrow adjacent zone, which corresponded to the limited diffusion of blood pigment from the trench to the adjacent medium (fig. 2).

This indicated that the system necessary to promote the sulphonamide action was present over the trench, to which enough sulphonamide readily diffused, whereas beyond the trench the limiting factor was the diffusion of the large hæmoglobin molecule or some substance equally limited in its range of diffusion.

The fact that a solution of crystalline horse hæmoglobin did not give this effect may be explained by supposing some interplay between the hæmoglobin solution and other elements present in hæmolysed blood which are removed in the course of dissolving the hæmoglobin crystals in N NaOH or on some subtle change in the

hæmoglobin molecule effected during the process. A solution of carefully washed red blood corpuscles was active, hence substances contributed by the serum were not important.

Influence of various components of the medium on the reaction

It was observed that there was considerable fluctuation in the development of the effect in different samples of medium. Occasionally it was very poorly developed, especially in recently prepared samples of nutrient agar ; more rarely a sample of nutrient agar was obtained in which, without the presence of horse blood, complete inhibition of growth was obtained in the vicinity of the trench with bacteria resistant to sulphonamide if inoculated on an ordinary sample of nutrient agar. Fig. 3 shows such effects when the different samples

Average sample of nutrient agar   Unusual sample of nutrient agar
as basis of heated blood agar   as basis of heated blood agar
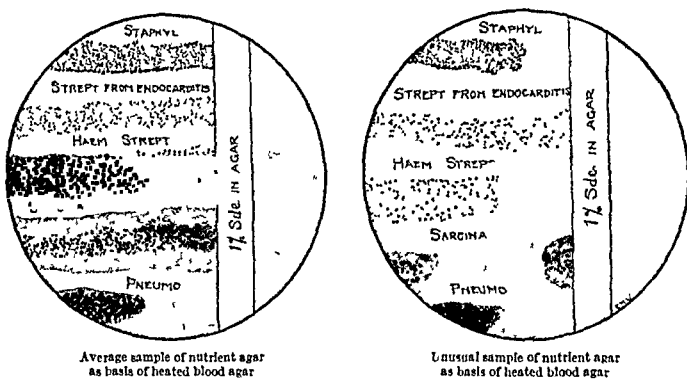
FIG 3 —Showing sulphonamide activity in an unusual sample of nutrient agar.

of agar are mixed with blood and the medium at once converted to "chocolate" by heating at 75° C.

It is to be noted that in this observation there was arrest of growth of a staphylococcus, a sarcina and a hæmolytic streptococcus on the unusual sample of nutrient agar only. Pneumococcus, which is a bacterium normally sensitive to sulphonamide in nutrient broth, was inhibited on both media ; but a *viridans* streptococcus from endocarditis was not inhibited on either.

A curious phenomenon was noted with the peroxide-forming sarcina, which was inhibited to a greater distance from the trench than the other bacteria but grew in the immediate vicinity of the trench. A possible explanation is that the sulphonamide was activated by some component of the medium, but that this action was weakened by diffusion to the non-nutrient agar in the trench and therefore had not the needed concentration in the immediate vicinity of the

trench to activate the sulphonamide, of which there was abundance. We cannot imagine how this can be interpreted in terms of the presence of a sulphonamide inhibitor in the medium. Such results suggested an investigation of the relative parts played by peptone and meat extract in promoting the sulphonamide effect.

In plates prepared with peptone agar only, we found that very little inhibition was developed in the vicinity of a sulphonamide trench, whereas with meat-extract agar and no peptone the effect was considerable, although a good deal less than that obtained in hæmolysed horse-blood meat-extract peptone agar.

In further investigations of the role of meat extract, plates were prepared containing peptone horse-blood agar with 0·5 per cent. sulphonamide in one half and nutrient agar in the other. When these were inoculated from side to side with a staphylococcus, growth resulted as shown in fig. 4.
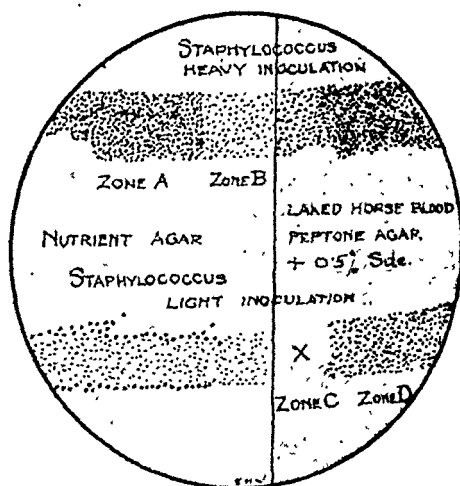
FIG. 4.—Showing the importance of meat extract. Complete inhibition of growth only where meat extract has diffused (×) into zone containing both hæmolysed blood and sulphanilamide.

Zone A shows good growth of staphylococcus in the absence of adequate sulphonamide concentration.

Zone B shows restricted growth where there is adequate sulphonamide diffusion and potentiation by elements in meat extract.

Zone C shows entire absence of growth due to the combined effect of horse blood and elements of meat extract, penetrating by diffusion, on the sulphonamide present.

Zone D shows good growth since, in the absence of elements of meat extract, the horse blood cannot promote sulphonamide action.

Observations.on the effect of horse blood on the action of sulphonamide diffusing into synthetic media

The advantage of synthetic media is that the possibility of the action of inhibitors of the sulphonamide effect is more limited and that the substances likely to produce the effect may be tested separately.

Two media were used. The first and more complex had the following composition : casein hydrolysate (acid) 2 per cent. ; K_2HPO_4 0·3 per cent. ; NaCl 0·1 per cent. ; cystine and ferrous ammonium sulphate each 0·005 per cent. ; $MgSO_4$ $7H_2O$ 0·004 per cent. ; nicotinic acid 0·001 per cent. These were dissolved and incorporated with 0·5 per cent. glucose in the requisite volume of 2·5 per cent. non-nutrient agar and brought to pH 7·4. The casein hydrolysate was prepared according to Mueller and Johnson's (1941) formula.

The bacteria tested were the Escherich strain of *Bacterium coli*, another strain of *Bact. coli* chosen at random from a fæcal culture used in routine work, and strains of *Shigella flexneri* and *Sh. sonnei*. All grew tolerably well on the medium ; and in the vicinity of a sulphonamide trench there was slight inhibition which was considerably accentuated if horse blood was incorporated in the medium (fig. 5).

Synthetic medium only

Synthetic medium+2% hæmolysed horse blood
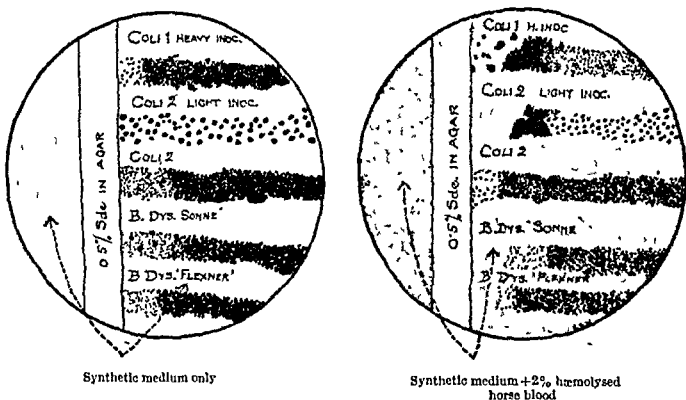
FIG. 5.—Showing increased sulphonamide effect when hæmolysed horse blood was added to a synthetic medium.

The second medium—C. M. MacLeod's 1940—was simpler, its only organic nitrogen being in the form of asparagin. On this medium as on the last there was slight inhibition of *Bact. coli* in the vicinity of the sulphonamide trench and much more marked inhibition when 2 per cent. of horse blood was incorporated in the medium.

Since MacLeod has shown that acid casein digest does not interfere with the sulphonamide effect, and since our own observations have established the same for nicotinic acid and asparagin, it is difficult to suppose that the increased sulphonamide effect with horse blood is due to removal of inhibitory substances. A potentiating effect seems a more probable explanation.

Nature of the substance or substances in broth which interfere with the action of sulphonamide

Since the work of Woods (1940) and Fildes (1940) *p*-aminobenzoic acid has received most attention amongst the inhibitors of sulphonamide (McIlwain, 1942, and many others), but it was excluded by Harper and Cawston since they were unable to obtain evidence that treatment with horse blood of broth containing added *p*-aminobenzoic acid removed its inhibitory effect on sulphonamide.

In view of the importance of this substance we also investigated the matter.

In each of our experiments a series of plates was prepared as follows: (1) 9 c.c. nutrient agar+1 c.c. horse plasma; (2) 9 c.c. nutrient agar+ 1 c.c. horse plasma+1 c.c. laked horse blood; (3) as (1)+1 : 1,000,000 *p*-aminobenzoic acid; (4) as (2)+1 : 1,000,000 *p*-aminobenzoic acid.

These were incubated for about 20 hours to allow full time for the Harper and Cawston effect to develop in respect of any inhibitory substances present in the medium at the outset, as well as the added *p*-aminobenzoic acid. A second similar series of plates was prepared next day, and both were immediately " ditched " with agar containing 0·5 per cent. sulphanilamide. All were then inoculated from side to side with two strains of staphylococcus and one strain of pneumococcus. The effects obtained in both series were practically identical, and only those of the first series are shown in fig. 6.

It would seem, therefore, that in a medium suitable for showing sensitivity of staphylococcus to sulphonamide the effect of the sulphonamide can be reversed by *p*-aminobenzoic acid, and that the influence of the *p*-aminobenzoic acid is not markedly counteracted by horse blood, as would be expected if the effect were due to a substance normally present in nutrient broth and responsible for the inhibition of sulphonamide action on the staphylococcus in such media. There was some evidence, however, that the *p*-aminobenzoic acid effect was diminished in the presence of horse blood and it is still possible to suppose that the Harper and Cawston effect is due to removal or masking of *p*-aminobenzoic acid if it is supposed also that the sulphonamide effect on staphylococcus is much more sensitive to *p*-aminobenzoic acid than the corresponding effect on pneumococcus. That is, one may suppose that nutrient agar contains traces of *p*-aminobenzoic acid sufficient to hold up the sulphonamide effect on staphylococcus but not on pneumococcus, and further that the horse blood (Harper and Cawston effect) can destroy or remove that amount of *p*-aminobenzoic acid but not the larger concentration used in our experiments.

If this is true, it should be possible to show that the sulphonamide effect on staphylococcus is more readily cancelled by *p*-aminobenzoic acid than that on pneumococcus. To test this point an experiment was carried out with agar based on Harper and Cawston filtrate and

therefore equally suitable for showing the Harper and Cawston effect on pneumococcus and staphylococcus. A series of plates was therefore set up to include a control containing "Harper and Cawston agar" alone
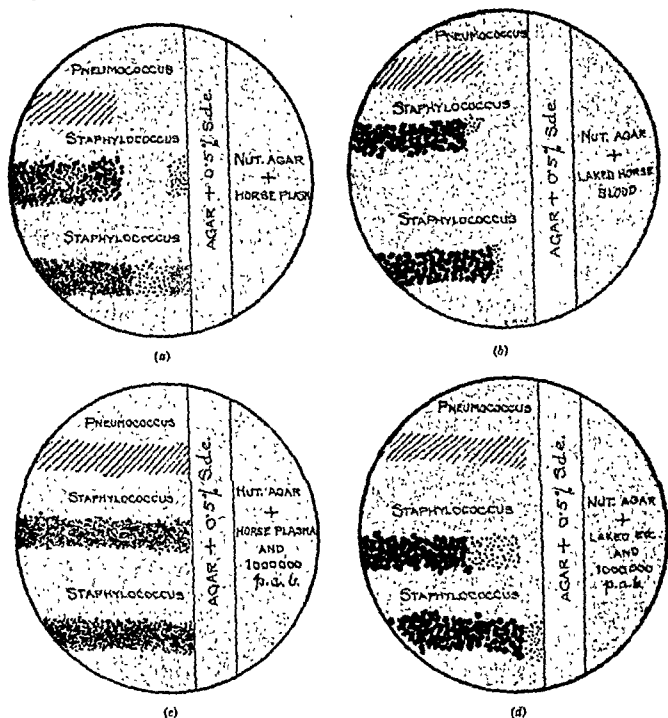
Fig. 6.—(a) Showing the usual inhibition of the pneumococcus in the vicinity of a sulphonamide trench and much more than the usual inhibition of the staphylococcus, probably due to traces of hæmoglobin which persisted in the plasma and gave a partial Harper and Cawston effect. The phenomenon already referred to—growth in the area adjacent to the trench but not at $\frac{1}{4}$-$\frac{1}{2}$ in. from it—was observed with one of the staphylococci.

(b) Showing complete elimination of staphylococcal growth for about $\frac{1}{2}$ in. from the trench—the usual Harper and Cawston effect.

(c) Showing complete or almost complete removal of the sulphonamide effect in the presence of p-aminobenzoic acid.

(d) Showing marked but incomplete removal of the sulphonamide effect by p-aminobenzoic acid.

(fig. 7a and b), and four other plates containing Harper and Cawston agar plus 1 : 250,000, 1 : 500,000, 1 : 1,000,000, or 1 : 10,000,000

p-aminobenzoic acid (fig. 7*c-f*).   Before the plates were poured 7 per cent. of horse blood was added to each tube and the whole heated at once for 10-15 mins. at 75° C. in order to provide a medium on which growth of the pneumococcus could be easily discerned.

The opposite half of the plate contained non-nutrient agar $+0.5$ per cent. sulphanilamide.   One series of plates was inoculated with pneumococcus and a second with staphylococcus.   The results are indicated in distances from the sulphonamide trench to which inhibition extended (table).

<div align="center">TABLE</div>

Inhibition of sulphonamide effect on staphylococcus and pneumococcus by different concentrations of p-aminobenzoic acid in " Harper and Cawston agar "

Plate no.	Concentration of *p*-aminobenzoic acid	Distance of growth inhibition from sulphonamide trench	
		Pneumococcus	Staphylococcus
1	Nil control	$\frac{3}{4}$ in.	$\frac{5}{8}$ in.
2	1 : 250,000	Growth up to trench; colonies a trifle smaller just beside trench	Growth up to trench but only minute colonies in last $\frac{1}{8}$ in.
3	1 : 500,000	$\frac{1}{8}$-$\frac{3}{16}$ in.	Marked check at $\frac{3}{16}$ in. but some very small colonies nearer trench
4	1 : 1,000,000	$\frac{1}{4}$ in.	Marked check to $\frac{1}{2}$ in. but minute colonies beyond
5	1 : 10,000,000	$\frac{3}{4}$ in.	$\frac{3}{8}$ in.

These results are reproduced in fig. 7.

It would seem, therefore, that *p*-aminobenzoic acid influenced the sulphonamide effect on both organisms to a like extent; it effected inhibition at 1 : 250,000 and had little action at 1 : 10,000,000. Other observations gave similar results.

From these observations it is clear that the presence of one part of *p*-aminobenzoic acid per million parts of medium would cut down the sulphonamide effect on the pneumococcus considerably; but since the pneumococcus is highly sensitive to sulphonamide in nutrient agar the *p*-aminobenzoic acid content of the normal sample of medium must be lower than that.   On the other hand, sulphonamide action on the staphylococcus is largely or entirely eliminated in the average sample of nutrient agar, an effect which would require at least one part per 500,000 of *p*-aminobenzoic acid.   Hence the inhibition of the sulphonamide effect on the staphylococcus observed in the average sample of nutrient agar cannot be due to *p*-aminobenzoic acid.

<div align="center">

Presence of inhibitory substances other than p-aminobenzoic acid in peptone

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The nature of the inhibitory action of peptones on sulphonamides has been discussed by McIlwain (1942), Sevag, Henry and Richardson (1943) and Kohn and Harris (1943) and others.   The last-named
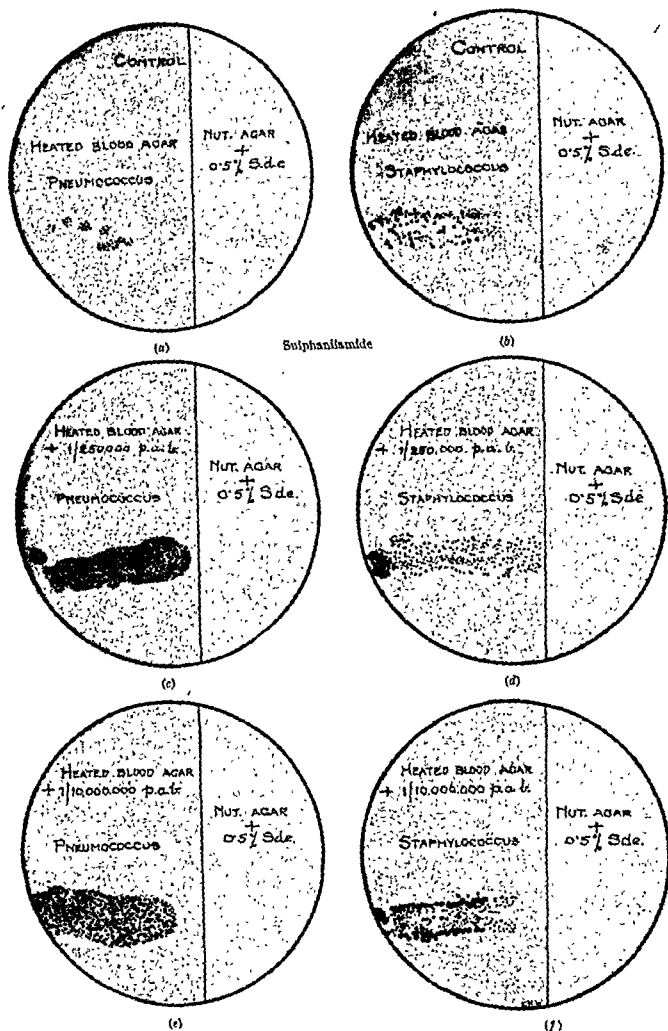
FIG. 7.—Showing that p-aminobenzoic acid neutralises sulphonamide action in a similar fashion for pneumococci and staphylococci.

All heated blood agar shown on this plate has a basis of Harper and Cawston broth.

sought widely for specific substances other than p-aminobenzoic acid giving this effect (Kohn and Harris, 1941) but the only one detected was methionine.

We have tested the effect on sulphonamide action of adding methionine to the Harper and Cawston filtrate agar, using the sulphonamide trench method described above and staphylococcus and Bact. coli as test organisms. We have failed, however, to demonstrate any distinct inhibitory action by this substance under these conditions.

Promotion of sulphonamide action on bacteria other than the staphylococcus in nutrient broth and agar containing horse blood

In their sensitivity to sulphonamides in nutrient broth or on agar, or such media enriched with blood or serum if this was required for the growth of the organism, it had previously been noted that the diphtheria bacilli and one diphtheroid tested, the pneumococci, and most notably the Neisseriæ, differed from most of the known pathogens (Burton et al., 1940).

The growth of such bacteria on plates trenched with sulphonamide agar was prevented for $\frac{1}{2}$ in. or more in the vicinity of the trench whether horse blood was incorporated or not, and a similar result was obtained in one experiment with Ps. pyocyanea. Among other bacteria which tended to grow right up to the margin of the trench on ordinary nutrient agar, on serum agar, on fresh blood agar prepared with blood other than equine, or on horse-blood agar heated at 75° C. within a few minutes of mixing ("chocolate" horse-blood agar), a considerable number showed restricted or completely inhibited growth in the vicinity of the sulphonamide trench on fresh horse-blood agar.

This effect was obtained notably with peroxide-forming hæmolytic streptococci and with a peroxide-forming sarcina. It was also observed with several strains of Bact. coli and with a strain each of Sh. shigæ, Sh. flexneri, and Sh. sonnei. It was poorly developed or failed completely with several strains of streptococcus, including a viridans strain from a case of endocarditis, and also with strains of Vibrio and Hæmophilus influenzæ; the latter were not, however, extensively investigated and were sometimes sulphonamide sensitive in the absence of horse blood.

Extent to which the phenomenon is obtained with various sulphonamides

Observations were limited to the sulphonamides which have been most extensively used in this country, i.e. sulphanilamide, sulphapyridine, sulphathiazole and sulphadiazine. Strains of staphylococcus and hæmolytic streptococcus, both of pathogenic origin, were investigated and comparisons were made (a) by determining the distance to which inhibition was obtained around a cup cut in horse-blood agar

and partly filled with a solution of the drug to be tested, and (b) by incorporating diminishing dilutions of the sulphonamide in horse-blood agar and determining which sulphonamide was active in highest dilutions. The cup tests indicated the order sulphanilamide<sulpha-pyridine=sulphadiazine<sulphathiazole. The tests with sulpha-thiazole were carried out extensively, both on the surface of horse-blood agar plates to which the drug was added and in hæmolysed horse blood and broth.

The results indicated that the efficiency of sulphathiazole in restricting the growth of staphylococcus was raised at least 1000-fold in the presence of horse blood, and that the more sensitive strains of streptococcus showed inhibited growth in concentrations ranging from 1 : 1,000,000 to 1 : 10,000,000 of sulphathiazole in the presence of horse blood. This means that under these conditions their growth was checked almost as much as that of the meningococcus and gonococcus, which are so outstanding in their sensitivity to sulphathiazole both *in vitro* and *in vivo*.

Does the increased sulphonamide effect developed in nutrient broth or agar in the presence of horse blood depend solely on the destruction of inhibitory substances or is the sulphonamide potentiated in some way ?

We have found it difficult to devise an experiment which will give an unequivocal answer to this question. It seems to us, however, that if the effect depends entirely on the removal of sulphonamide inhibitors, treatment with horse blood must remove them, either completely or partially. If completely, concentration of the filtrate should make no difference ; if partially, there should be inhibition of the sulphonamide effect in proportion to the concentration of the filtrate.

When experiments were made to test this, the results seemed to point simultaneously to the occurrence of the destruction of substances inhibitory to sulphonamide action and to the existence of some independent sulphonamide-potentiating system. The results obtained are illustrated by the three photographs of plate cultures in figs. 8, 9 and 10. In fig. 8 the left side of the plate contains non-nutrient agar+0·5 per cent. sulphonamide, the right side an agar prepared by mixing equal parts of a Harper and Cawston filtrate and 5 per cent. non-nutrient agar. Three dilutions with decreasing numbers of staphylococci were inoculated right across the plate. There is good growth over the right side in the part remote from sulphonamide diffusion, absence of growth in the usual zone of sulphonamide diffusion into the right half from the opposite section of the plate, and no growth over the non-nutrient agar. In fig. 9 the left side of the plate contains nutrient agar+0·5 per cent. sulphonamide, the right side as in fig. 8. The result shows growth on the right side as in fig. 8,

and also over the whole of the left side and to some distance over the right side in the vicinity of the junction, but not in a zone $\frac{1}{4}$-$\frac{1}{2}$ in. distant from the junction.

This result would fall in completely with Harper and Cawston's theory. Growth takes place in nutrient agar+sulphonamide because of the sulphonamide inhibitors present, and also on the right side on account of diffusion of these inhibitors into the Harper and Cawston filtrate agar which has been freed from them, but not in a zone $\frac{1}{4}$-$\frac{1}{2}$ in. from the junction because they do not diffuse so rapidly as to reach that area in which the more rapidly diffusing sulphonamide is present in adequate concentration to inhibit growth.

The third plate culture (fig. 10) differs from the second in containing on the right side an agar prepared by mixing non-nutrient agar with a Harper and Cawston filtrate five times concentrated by vacuum distillation at temperatures less than 50° C. Here the zone of growth on the right side near the junction is completely obliterated and there is a slight indication of inhibition of growth on the left side of the plate in the area nearest the junction. If all that had happened to the Harper and Cawston filtrate was removal of inhibitors, this result could not have been obtained. Since the growth on the part of the right side of the plate remote from sulphonamide penetration is stronger than in the plates shown in figs. 8 and 9, the explanation of this failure must be either a potentiation of sulphonamide under the influence of concentrated Harper and Cawston filtrate, or the development in it of some system capable of destroying additional sulphonamide inhibitors. No similar effect was obtained when the right half of the plate was prepared by mixing a five-fold concentration of ordinary broth and non-nutrient agar.

CONCLUSIONS

1. Staphylococci and several other bacteria, including many strains of hæmolytic streptococci, are relatively insensitive to sulphonamides in the average sample of nutrient broth or agar.

2. In the presence of horse blood, especially hæmolysed horse blood, these bacteria develop marked sensitiveness to sulphonamides (Harper and Cawston effect).

3. The hæmoglobin of blood or something closely associated with it or a combination of these appears to be responsible for the effect.

4. The effect has not been obtained in a marked degree with blood other than equine.

5. The effect is not due to destruction of p-aminobenzoic acid or of methionine.

6. After nutrient media are incubated with horse blood, steamed and filtered, a fluid is obtained which no longer inhibits sulphonamide action.

7. It is probable that potentiation of sulphonamide plays a considerable part in this change, although elimination of inhibitory substances may also occur.

BACTERIAL SENSITIVITY TO SULPHONAMIDES
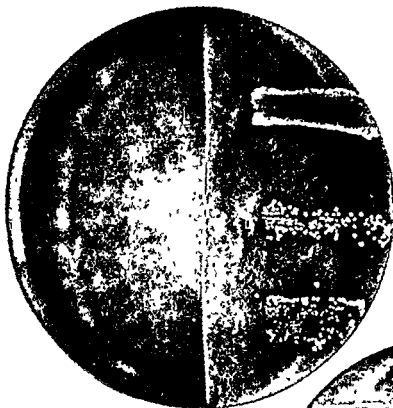
FIG. 8.—Left half contains non-nutrient agar + 0·5 per cent. sulphonamide; right half contains Harper and Cawston filtrate in 5 per cent. non-nutrient agar. Three dilutions of staphylococci inoculated across the plate. Note absence of growth in zone of sulphonamide diffusion into right half of plate.

FIG. 9.—Left half contains nutrient

$\frac{1}{4}$-$\frac{1}{2}$ in. from junction of two halves of plate.
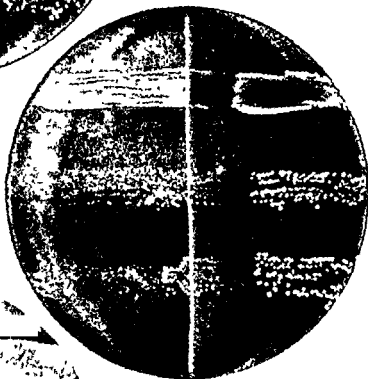
FIG. 10.—Left half as in fig. 9; right half as in figs. 8 and 9 except that Harper and Cawston filtrate has been concentrated five-fold. Inoculum as in figs. 8 and 9. Note that growth on right side near junction is obliterated and that growth is slightly inhibited on left side nearest the junction.

8 Evidence for potentiation of the sulphonamide is derived from —(a) the influence of horse blood in synthetic media in which no inhibitory substance can be demonstrated, (b) the increase of the sulphonamide effect in concentrated filtrates obtained by Harper and Cawston's method

REFERENCES

BURTON, H , McLEOD, J W ,   1940   *Brit J. Exp Path* , xxi, 288
   McLEOD, T S , AND MAYR
   HARTING, ANNA
FILDES, P   1940   *Lancet,* i, 955
HARPER, G J , AND CAWSTON,   1945   This *Journal,* lvii, 59
   W C
KEILIN, D , AND HARTREE, E F   1935   *Proc Roy Soc* , B, cxvii, 1
KOHN, H I , AND HARRIS, J S   1941   *J Pharm and Exp Therapy*, lxxiii, 343
   ,,   ,,   ,,   1943   *Ibid* , lxxvii, 1
McILWAIN, H   1942   *Brit J Exp Path* , xxiii, 265
MACLEOD, C M   1940   *J Exp Med* , lxxii, 217
McLEOD, J W , MAYR HARTING,   1944   This *Journal*, lvi, 377
   ANNA, AND WALKER, N
MUELLER, J H , AND JOHNSON,   1941   *J Immunol* , xl, 33
   E R
SEVAG, M G , HENRY, JANE, AND   1943   *Amer J Med Sci* , ccv, 877
   RICHARDSON, RUTH
WOODS, D D   1940   *Brit J Exp Path* , xxi, 74

A CASE OF FŒTUS PSEUDO-AMORPHUS

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(PLATE XCVIII)

PSEUDO-AMORPHOUS fœtuses are rare monstrosities and are always worthy of description. References to some related examples and other relevant literature are given at the end of this account.

The clinical history in the present case is rather short. This was the first pregnancy of a woman aged 20 years who suffered from mitral stenosis. The pregnancy was not abnormal and the fœtus, a single one, was delivered at term.

DESCRIPTION OF SPECIMEN

External appearance (figs. 1 and 2)

The fœtus measures $17 \times 9 \times 5$ cm. Its weight is 564 g., its shape an ovoid somewhat flattened in the dorso-ventral direction. A deep cleft near the middle of the specimen divides it into an upper ovoid and a lower rounded part (fig. 2). The upper portion is partly covered with short hairs. The skin here is very tough, in contrast with that of the lower part, which is smooth and soft. The fœtus presents dorsal and ventral surfaces. The principal feature of the ventral surface of the upper part (fig. 1) is a flat triangular formation, 3 cm. long and 2·2 cm. wide, which corresponds to the nose. Near the base of the triangle are two oblique, blindly-ending openings—rudiments of the nostrils. On all sides the nose is surmounted by folds of skin of varying height. Two of these are prominent and cover the eyes. Beneath the nose is a small funnel-like cavity lined by soft skin, at the bottom of which a small aperture communicates with two tubes—the œsophagus and the trachea. The nose is flush with the surrounding skin surface. The umbilical cord projects from the ventral surface of the lower part of the fœtus. It has two vessels only, an artery and a vein. The skin here is smooth and without folds. On the dorsal aspect of the lower part are two rudimentary lower limbs, each 5 cm. long, with many folds of skin and spade-like

enlargement of their terminal parts. Each limb, divided from the remainder of the trunk by deep lateral clefts, is quite flat and ends in five very rudimentary toes on the left side and four on the right. The limbs are turned upwards and are tightly applied to the surface of the body; between them there is situated a trunk-like prominence which represents the external genitalia (fig. 2). Under this prominence is a small anal opening. The limbs and genital fold occupy nearly the whole of the dorsal surface of the lower third of the fœtus. The dorsal surface of the upper two-thirds is fairly smooth, but with areas bearing fine hairs; a thick fold of skin demarcates it from the lower part. The sides of the upper part of the fœtus bear two rudimentary upper limbs. The left, which is 2·4 cm. long and 2 cm. wide, has a conical shape. It carries four fingers, one of which is in opposition to the other three. The terminal parts of the fingers carry small nails, and the second finger is broader than the others. The right upper limb consists only of three long fingers. It is 1·5 cm. long and 0·5 cm. wide.

The skeleton (fig. 3)

The skull is high and rather narrow. Maxilla and mandible are absent and thus the nasal cartilage projects forward prominently. The distal border of the squamous part of the frontal bone bears a prominent superciliary ridge. The orbital parts of the frontal bone make an angle of 145° with the inferior margin of the squamous part. The lateral portions of the orbital parts form very small zygomatic processes, more prominent on the right than on the left side. The lateral, distal and medial boundaries of the orbital parts are formed by fibrous tissue, connecting the orbital parts with the cartilage of the nose and with the sphenoid bone. These orbital parts of the frontal bone are the only bones taking part in the formation of the roof of the orbit. They are quite flat.

Extensive thick fibrous membranes, which take the place of the parietal bones, connect the frontal with the occipital bone, which consists of a squamous and a basilar portion. On the squamous part is a large and high planum occipitale, the distal boundary of which forms a conspicuous external protuberance. From this there extends laterally on each side a semicircular superior nuchal line, below which the bone shows a planum nuchale. Below the external occipital protuberance the squamous part of the occipital bone is replaced by a long strip of cartilage. This has the shape of two inverted horse-shoes connected in the middle. Caudalwards from the cartilage there extends a fibrous membrane attached on each side to two bony bars which join ventrally and correspond to the basilar part of the occipital bone. Thus the foramen magnum has normal boundaries. The hypoglossal canals are present in the basi-occiput. Occipital condyles are absent.

Fig 1 —Fœtus pseudo amorphus central aspect On the left above the centre, there is a flat triangular formation corresponding to the nose × ½ (c)

Fig 2 —Fœtus pseudo amorphus, dorsal aspect × ½ (c)

Fig 3 —Skull and spine Note prominent nasal cartilage with two blindly ending tube like for mat ons and the exophthalmic position of the eye × ½ (c)

Fig 4 —General view of viscera of head and trunk showing brain heart with the common auricle and common arterial trunk and its branches, left pulmonary artery and left lung, liver, etc × ½ (c)

A square block of cartilage connects the basilar part of the occipital bone with the sphenoid, which consists of body and wings only. Viewed from above there is a small sella turcica, overhung posteriorly by a very low dorsum sellæ. The posterior clinoid processes are absent. In front of the sella turcica a large deep median fissure partly filled by cartilage divides the body of the sphenoid into two parts which project up and form the anterior clinoid processes. Laterally two wings project from the body of the sphenoid. Greater and lesser wings cannot be distinguished, but a prominent optic foramen is present in each wing and dorsally each wing carries a small prominence directed towards the anterior clinoid process. In this way the foramen ovale is enclosed. The sphenoid is in a nearly vertical position and the part of the body which carries the sella turcica forms a small square plate under the nasal cartilage. The pterygoid processes are absent.

On each side the temporal bone is very poorly developed and it is difficult to identify its parts. Most prominent is the squamous part, in the middle of which there is a large irregular aperture around which the bone shows many small fissures and prominences. On the distal edge of the squamous part is a region of thicker bone which from the inside appears divided from the squamous part by a deep fissure. On this thick part we may distinguish a back portion which corresponds to the mastoid process and a front portion which corresponds to the petrous part. The blindly ending internal auditory foramen is situated in this part. There are many excavations and prominences on the surface of the temporal bone. The styloid and zygomatic processes and the external auditory meatus are absent.

In the region of the nose are some small bones which are connected with the triangular nasal cartilage. On the upper edge of the cartilage, which is connected by fibrous tissue with the frontal and sphenoid bones, are two small bones corresponding to the nasal bones. In the ventral part of the nasal cartilage are two blindly ending tube-like formations each with a small bony plate in its roof. It is difficult to identify these bony plates, but perhaps they are rudimentary turbinates. On the distal edge of the nasal cartilage is a long narrow bony plate which corresponds to the vomer. On both sides of the nasal cartilage are fibrous membranes forming two pocket-like formations, on the outside of which there are two small bones corresponding to lachrymal bones. Inside this pocket there is a fibrous fold containing a small bone, probably the inferior turbinate.

The spine is very poorly developed. It consists of a solid cartilaginous column 4·3 cm. long, divided by two lateral grooves into anterior and posterior parts. The latter has a gutter-like shape and is connected by a fibrous membrane with the basilar part of the occipital bone. The anterior part is rounded and is connected directly to the basilar part of the occipital bone. The segmentation of the vertebral column is imperfect. Small centres of ossification are

present in the posterior part, six on the left side, five on the right, and small cartilaginous prominences are present corresponding to spinous processes. The spine is slightly sinuous, its lower part being concave anteriorly.

The limbs are so grossly malformed that it is difficult to recognise and identify their component bones. The skeleton of each lower limb is represented in its upper part by a rectangular mass of cartilage connected with an angulated bone 1·5 cm. long. This cartilage and bone are attached to the spine only by fibrous tissue ; they possibly represent rudiments of the pelvic girdle. At its distal end the bone is joined by a nodular mass of cartilage to another thick triangular bone, corresponding perhaps to a malformed femur. The femur is joined to a thick nodular mass of cartilage which is connected distally with well developed metatarsal and phalangeal bones.

The skeleton of each upper limb is represented only by long thin metacarpal and phalangeal bones. There are four sets of digital bones on the left side and three on the right.

From the region of the upper limbs some cartilaginous strips run ventralward and join together just above the pericardial cavity. These strips are rudiments of the ribs.

The muscular system

Some groups of muscles are well developed, some of them show major abnormalities. The neck muscles are developed best of all. They take origin from the occipital bone and the distal edge of the temporal bone, and run caudalward to the rudimentary spine and upper limbs. Sternomastoid, splenius capitis, splenius cervicis, scaleni and trapezius muscles can be identified on both sides, but other muscles are not identifiable. Distally all these muscles are connected with a broad layer of undivided muscular tissue which runs the whole length of the specimen and passes from its dorsal attachments in a ventral direction. This sheet of muscle lies immediately under the œdematous subcutaneous tissue. On the dorsal side of the fœtus at a level just below the well developed diaphragm and situated on each side of the spine there is present a thick and prominent muscle which runs caudalward and joins the cartilage of the posterior limb. This muscle probably corresponds to the quadratus lumborum. Prominent on the dorsal aspect of the fœtus is a short thick muscle arising from the end of the spine and running obliquely caudalwards also to this cartilage. It is probably the psoas muscle. Below the cartilaginous part of the presumed pelvic girdle the muscular sheet forms a semicircular structure of increased thickness. The limbs contain muscles most of which, however, cannot be specifically identified. The best developed are the interossei of both hands and feet.

The circulatory system (figs. 4 and 5)

The heart, 2 cm. long and 1·5 cm. wide, is situated in a pericardial sac. From the base arises only one vessel—an arterial trunk. On the right side there is a large elongated auricle which overlaps the arterial trunk ventrally. There is no separate left auricle. The auricle merges distally into a large thin-walled sac forming the whole dorsal wall of the heart. The distal part of this common auricle is widely open and funnel shaped. Here is situated the aperture of the inferior vena cava, underneath which the wall of the atrium is raised in the form of a high thin fold corresponding to the Eustachian valve. In the upper part of the atrium is the aperture of the superior vena cava. The atrial septum is absent and on the left side of the common cavity are the openings of the pulmonary veins. The coronary sinus is absent. The common arterial trunk arises from the part of the heart which is clearly the persistent bulbus. This has the shape of a short and narrow canal, its walls formed by the muscular tissue of the heart, which here becomes thickened and cushion-like. Below the bulbus is the large cavity of the right ventricle, and an incomplete interventricular septum separates this chamber from the narrow left ventricle, which has a slit-like cavity. The right ventricle communicates by a wide aperture with the large common atrium. The interventricular septum passes upwards in an oblique direction beneath the left muscular cushion of the persistent bulbus towards

Fig. 5 —Anatomy of heart, etc. (1) Inferior vena cava (2) Orifice of inferior vena cava (3) Wall of common atrium (4) Right auricle. (5) Cavity of common atrium (6) Tricuspid valve. (7) Interventricular septum. (8) Bicuspid valve (9) Wall of heart. (10) The cushion like formation in bulbus (11) Persistent bulbus. (12) Orifice of superior vena cava. (13) Valves of common arterial trunk. (14) Left coronary artery. (15) Common arterial trunk. (16) Left pulmonary artery. (17) Orifices of pulmonary arteries. (18) Right pulmonary artery. (19) Innominate artery. (20) Left common carotid artery. (21) Left subclavian artery. (22) Descending aorta. ×2 (c.)

the lateral wall of the heart. In the membranous part of the septum a semicircular defect is present and at this point are found the attachments of the medial and anterior cusps of the tricuspid valve. A membrane which represents the poorly developed bicuspid valve

and is fused with the interventricular septum nearly closes the aperture of the left ventricle.

The main arterial trunk has an ascending part, an arch, and a descending part. Dissection shows that there is no division into aorta and pulmonary artery. The root of this common vessel possesses four semilunar valves, and 1 mm. above these, in the middle of the posterior wall of the vessel, there is the small orifice of the left coronary artery. This vessel runs obliquely from the right side of the anterior wall of the heart towards the apex. At a level 1 mm. above the orifice of the coronary artery there are the two closely set small orifices of the pulmonary arteries, which pass to the well developed lungs. From the lateral wall of the common vessel at the level of the pulmonary arteries the innominate artery arises and opposite it the left common carotid artery takes origin in an oblique direction. These arteries thus form a U-around the œsophagus and trachea. The left subclavian artery arises 0·5 cm. from the left common carotid artery —nearly at the end of the arch. The part above the pulmonary arteries is the aortic arch and the part caudal to the left subclavian artery is the descending aorta. All the latter's parietal and visceral branches arise as in the normal.

No signs of a lymphatic system could be found, but it should be mentioned that three large cavities filled with clear yellow fluid are present in the dorsal region of the specimen. Œdema of the subcutaneous tissues is also present.

The respiratory system

A larynx is not differentiated and the respiratory system in its upper part is a simple tube which opens directly into the rudimentary mouth cavity and is separated from the œsophagus at its upper end only by a thin fibrous septum. Lungs and pleuræ are well developed. The right lung consists of two lobes of which the upper is divided into three small prominences by two short clefts running caudalwards from the apex. The left lung is abnormal in that its apex forms a pyramidal prominence which passes in a ventral direction.

The digestive tract

As a result of failure of development of the maxilla and mandible an oral cavity is absent and in its place there is a funnel-like aperture situated beneath the nose and lined with soft skin. The digestive tract begins with a simple opening at the end of the mouth funnel and passes immediately into the œsophagus. Stomach and small intestine are present and appear normal. The colon is abnormal, being very short—only 2·5 cm. long—without any differentiation into its component parts. It passes in a ventro-dorsal direction in the space between the elements of the pelvis and ends in a small anus just

below the rudimentary external genitalia. The colon is dilated and its walls are thin, this dilatation being due to compression by the muscles and bones of the lower limbs. The liver is large and normally situated but not lobulated. Gall bladder and pancreas are present and appear normal.

The urogenital system

Only the shape of the kidneys is abnormal. They are round and flat and the ureters run on their ventral surfaces. They are situated opposite the lower end of the spine on the dorsal wall. On the lateral aspect of each kidney there is a small genital gland. Microscopical examination shows that these are rudimentary testes. On the upper pole of each is a minute drop-like formation corresponding to the epididymis. The ductus deferens forms a thin cord running caudalward and becoming lost in the soft fibrous tissue of the peritoneum at the level of the urinary bladder. The seminal vesicles, bulbourethral glands, prostatic gland and scrotum are absent.

The endocrine glands

All these are normally developed with the exception of the suprarenals, which form a single large horseshoe-shaped yellow mass situated between the kidneys.

The nervous system (figs. 3 and 4)

Although the brain and spinal cord are well developed and their main parts can be identified, fixation is so bad that their precise description is impossible. Some of the cranial nerves can be distinguished, namely the olfactory and optic nerves and the vagus. Many spinal ganglia are present and spinal nerves run through the lateral fissures in the cartilaginous column of the spine to reach the muscular tissue of the dorsal part of the fœtus. The spinal cord ends in a formation similar to the cauda equina. No trace of a sympathetic nervous system is found.

Because the orbital part of the frontal bone and the sphenoid together form an almost vertical wall the optic foramen is situated very low. It is placed at the level of the distal edge of the nasal cartilage and the ocular muscles and optic nerve pass upwards to reach the eye. After removal of the skin the eyes are very prominent because they are situated on this vertical optic part of the frontal bone. The eyeball is normally developed.

MICROSCOPIC EXAMINATION

This was unsatisfactory because of poor fixation, but as far as could be ascertained the structure of all the main organs appears substantially normal.

DISCUSSION

In this specimen, in spite of the poor development of its externally amorphous features, many organs and systems are well developed. It is therefore necessary to designate the specimen as a foetus pseudo-amorphus. It cannot be included in the group of hemiacardiac foetuses because these monsters, like the acardiac foetuses, are always twins, while the present monster is a single foetus with its own well developed circulatory system. The most notable changes are in the skeletal, muscular and circulatory systems, and are such as to suggest early arrest in the development of the segmentation of the sclerotomes and myotomes. This idea is especially supported by the presence of the poorly segmented cartilaginous spine. It seems probable that the development of the spine was arrested at the stage of primitive pre-cartilaginous mesenchymal tissue, corresponding to about the 4th week of development (Keith, 1933 ; Florian and Frankenberger, 1939). That this arrest of segmentation involved not only the sclerotomes but also the myotomes seems to be supported by the presence of a large undifferentiated muscular layer in the thoracic and abdominal region. The primary change which caused these malformations resulted also in abnormal development of the skull, the pelvic girdle and the upper and lower limbs.

The above hypothesis differs from that put forward by many authors with regard to hemiacardiac and acardiac (amorphous) foetuses. These are usually regarded as the consequence of malformation of the circulatory system, the defective foetus being considered as a parasite on the circulatory system of the second foetus—the chorionangiopagus parasiticus (Schwalbe). The fact that the present specimen is a single foetus with a circulatory system of its own supports the idea now put forward.

It is probable that the poor circulation due to the primary cardiovascular malformations occasioned secondary changes due to mechanical factors. The saccular dilatation of the common atrial cavity, the funnel-like formation of the orifice of the inferior vena cava, and perhaps also the formation of the large atrioventricular ostium may be regarded in this way.

On the other hand the persistence of the truncus arteriosus and related malformations of the heart found in the present case have been described in foetuses and children which were otherwise normally developed (Humphreys, 1932 ; Kettler, 1939 ; Hunter, 1944 ; Webb, 1946). Because of this, it is possible that the malformation of the heart in the present case may be independent and related only coincidentally to the other abnormalities, but the idea that they are related seems the more probable. However, this question and that of the causal genesis of the malformation of the basic mesenchyme must be left without final answer.

SUMMARY

A fœtus pseudo-amorphus, born of a mother suffering from mitral stenosis, is described. It is a single fœtus, and the most interesting changes are found in the skeleton, muscles and circulatory system, especially the heart and aorta. The malformation is interpreted as the result of early disturbance in the segmentation of the sclerotomes and myotomes and in the formation of the heart.

REFERENCES

FLORIAN, J., AND FRANKEN-   1939.   Embryologie, *Prague.*
BERGER, Z.
HUMPHREYS, ELEANOR M.   .   .   1932.   *Arch. Path.*, xiv, 671.
HUNTER, O. B., JR.   .   .   .   .   1944.   *Ibid.*, xxxvii, 328.
KEITH, A.   .   .   .   .   .   .   1933.   Human embryology and morphology, 5th ed., *London.*
KETTLER, L. H.   .   .   .   .   1939.   *Arch. path. Anat.*, ccciv, 513.
WEBB, A. C.   .   .   .   .   .   1946.   *Arch. Path.*, xlii, 427.

ADDITIONAL REFERENCES

BRODSKY, I.   .   .   .   .   .   1939.   *Med. J. Austral.*, i, 539.
BUXBAUM, H., AND WACHSMAN,   1938.   *Amer. J. Obst. Gyn.*, xxxvi, 1055.
D. V.
DIETRICH, A.   .   .   .   .   .   1906.   *Verh. Dtsch. path. Gesel.* (1905), ix, 198.
GRÖGLER, F.   .   .   .   .   .   1933.   *Arch. path. Anat.*, cclxxxix, 430.
HAMILTON, W. J., BOYD, J. D.,   1945.   Human embryology, *Cambridge.*
AND MOSSMAN, H. W.
HOLTERMANN, C.   .   .   .   .   1927.   *Med. Klin.*, xxiii, 460.
KAPPELMAN, M. D.   .   .   .   1944.   *Amer. J. Obst. Gyn.*, xlvii, 412.
LE VINE, I., AND WOLF, I. J.   .   .   1940.   *Ibid.*, xl, 327.
SCHMID, B.   .   .   .   .   .   1936.   *Z. Geburtsh. Gynäk*, cxiii, 272.
SEIDL, J.   .   .   .   .   .   .   1927.   *Sborník lékařský*, xxix, 109.
SIMONDS, J. P., AND GOWEN, G. A.   1925.   *Surg. Gyn. Obst.*, xli, 171.
STEWART, M. J.   .   .   .   .   1913-14.   *Proc. Roy. Soc Med.*, vii, Sect. Obst. and Gyn., 131.
VAN TONGEREN, F. C.   .   .   .   1932.   *Zbl. Gynäk*, lvi, 594.

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BACTERIAL "CALCULI"

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(PLATES XCIX-CI)

THE production of calcium carbonate by micro-organisms receives only scanty reference in textbooks of bacterial chemistry. Buchanan and Fulmer (1928), for example, refer only to the formation of calcium carbonate crystals on the sporangia of certain species of Mucor, while Brussoff (1916, cited by Buchanan and Fulmer, 1930) records the precipitation of calcium carbonate as a white coherent film by *Ferrobacterium calceum*. The formation of visible aggregations of calcium carbonate by commonly encountered varieties of bacteria growing on routine solid media does not appear to have been described. Since this process may give rise to curious colonial appearances liable to be ascribed to bacterial dissociation, an account of the phenomenon may be of interest.

Preliminary observations

In the course of a routine bacteriological examination a pure growth of *Bact. friedländeri* was obtained from the sputum of a patient who had been on penicillin inhalation therapy for two weeks. Examination of individual colonies with a hand lens revealed a number of small white particles lying within the translucent colony substance and distinguishable from it by their greater density. Examination under the low power of the microscope confirmed the discrete nature of the particles, which displayed a certain uniformity in shape but considerable diversity in size. These bodies were situated on the surface of affected colonies, which showed interruptions of their normal gloss when viewed by oblique reflected light. Stained films of these colonies showed uniform Gram-negative bacilli; there was no evidence to suggest that the intra-colonial bodies were symbionts. In view of these findings, the appearances were at this time considered to be a manifestation of bacterial dissociation, and attempts were made, using a micromanipulator, to subculture the opaque bodies. In every case the subcultures gave normal *Bact. friedländeri* colonies, in which, however, the formation of these intra-colonial bodies was repeated. In the course of the next few days all the cultures grown

at the routine bacteriology bench were examined under the low power of the microscope, and a similar condition was observed affecting colonies of a wide variety of organisms including *Proteus vulgaris, Bact. coli, Bact. typhosum, Staphylococcus aureus, Ps. pyocyanea, Bacillus subtilis* and *Corynebacterium hofmannii*; the appearances were not seen on smaller colonies such as those of streptococci and pneumococci. One exceptionally moist culture of Proteus provided incontestable evidence that the bodies were intra-colonial and had not merely formed in the underlying agar, for in this culture they could be seen streaming about in the convection currents present in the growth.

The composition of these particles is described in greater detail below; it suffices here to state that they contain a high proportion of calcium carbonate. The appearances described are reproduced in figs. 1-3. Fig. 1 shows a culture of Proteus on blood agar photographed by oblique reflected light against a dark background. The white areas are zones in which intra-colonial particles have formed. Fig. 2 shows the appearance of the particles under the low power of the microscope. The structure of the bodies is seen under higher magnification in fig. 3.

Cultural conditions

It became clear from the wide variety of organisms displaying the phenomenon that it was dependent upon a peculiarity of the medium. Now the medium on which these appearances had been produced was of standard composition: 2·5 per cent. agar fibre, 1 per cent. Lemco (Oxo), 1 per cent. Evans's peptone, 0·5 per cent. sodium chloride and 3 per cent. oxalated horse blood, these constituents being made up in tap water. Phosphates were precipitated by boiling and removed by filtration, and the medium was clarified by filtration through paper pulp and acid-washed sand. In spite of these standard constituents and the routine method of preparation, it was found that the phenomenon would occur with one batch of medium and not with another.

Attempts were next made to reproduce the unusual colonial appearances by adding various substances to a simple beef infusion nutrient agar made up with distilled water. It was found that the appearances could be reproduced consistently by the addition of soluble calcium salts. A series of plates was prepared from this simple medium containing concentrations of superadded calcium ranging from 0·1 to 1·0 per cent. (approximately). Each plate was inoculated with a coliform organism which had produced carbonate particles previously and was incubated for 24 hours. A good growth was obtained on all plates, no suggestion of inhibition being seen in any plate. The cultures on media containing 0·3 per cent. or less of added calcium showed no carbonate bodies. The cultures on media containing higher concentrations, up to and including 1 per cent.,

PLATE XCIX

BACTERIAL " CALCULI "
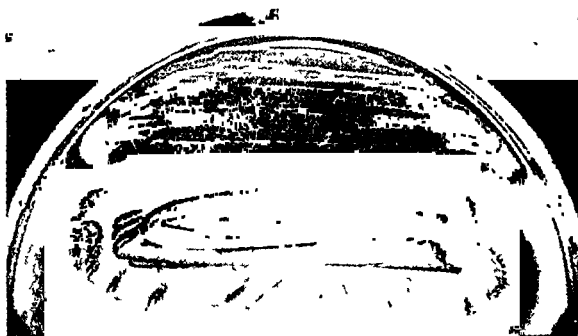
Fig 1—Carbonate bodies in a culture of Proteus   ×1 2
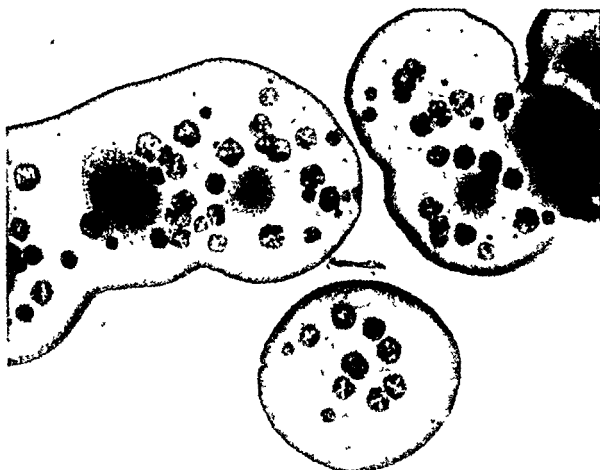
Fig 2—Intra colonial carbonate bodies   ×30

formed numerous carbonate bodies, though their number and size showed no tendency to increase with the concentration of calcium. Other soluble calcium salts such as calcium acetate were as effective as calcium chloride, but relatively insoluble salts such as calcium oxide were ineffective. Part of the added calcium is precipitated as calcium phosphate and a further part is probably bound to protein, when it may or may not be available to form carbonate. Should calcium so bound be unable to take part in the reaction, then clearly a proteolytic organism may make it available. In this connection it may be mentioned that proteolytic organisms such as *Ps. pyocyanea* and Proteus show a special facility in the formation of carbonate on a medium containing a relatively low concentration of calcium. Precipitation of calcium as phosphate and binding with protein make it almost impossible to state the minimum concentration of "free" calcium required for carbonate formation to occur The range of calcium concentration with which the phenomenon was observed, *i.e.* between 0·3 and 1·0 per cent. of calcium chloride, was considerably below the inhibitory concentration given by Winslow and Hotchkiss (1921-22) for *Bact. coli* in 1 per cent. peptone, namely 0·5 *M* (5·5 per cent.).

The appearance of carbonate bodies in cultures growing on routine media is evidently due, when it occurs, to a fortuitously high calcium content. The constituents of the Lab Lemco medium referred to were tested qualitatively for the presence of calcium. It was found that solutions of peptone, of Lab Lemco and of agar fibre in distilled water all gave moderate precipitates on the addition of ammonium oxalate solution. It is concluded that the variability in the formation of calcium carbonate bodies from one batch of medium to another is due to variation in the calcium content of one or more of the constituents of the medium. The source and physical properties of agar are such that a variation in the calcium content might well be expected.

Drying of the culture is not a factor in the formation of carbonate bodies : they have been produced in overnight cultures growing in screw-capped vials containing condensation water.

The distribution of carbonate bodies in a culture is seldom uniform : in an area of confluent growth the bodies are most evident in a zone immediately within the periphery, and in general it may be said that they tend to be most concentrated over the thickest parts of the culture where, presumably, metabolism and reproduction have been greatest. Sometimes the bodies tend to be concentrated in the cultures over the scratches made by the inoculating loop. When colonies are discrete but close together, the bodies may be seen only in the colonies towards the periphery of the plate. This may be due to the greater share of nutrients available to the peripheral colonies and their consequent greater metabolic rate.

Composition of the carbonate bodies

When an emulsifiable culture containing carbonate bodies is shaken up with water, the bodies retain their discrete nature and sediment rapidly. It is therefore fairly simple to wash them free of culture and to subject them to simple physical and chemical examination.

It was found (using polaroid accessories) that they were optically active and insoluble in water and organic solvents. They charred slightly on heating but retained their form and optical activity on heating to the melting point of glass. They dissolved incompletely in dilute mineral acids with the evolution of carbon dioxide and the resulting solution gave a heavy precipitate with ammonium oxalate solution. The fraction remaining after the action of acid was studied by observing the acid digestion of an isolated particle through the microscope. The optical activity was slowly discharged from without inwards, leaving an optically inactive " ghost " of much the same size and shape as the original particle. When acid digestion was arrested by dilution after it had proceeded half-way through a particle (as observed by polarised light), and the specimen then fixed by heat, stained with methylene blue and examined under oil immersion, the preparation revealed that the optically inactive periphery consisted of bacteria. These were somewhat pleomorphic and irregularly stained. The optically active fragment remaining in the centre of the particle still contained closely packed bacteria which could be focussed at various levels throughout the fragment. These enmeshed bacteria showed very little capacity to take up the stain, evidently on account of the barrier provided by the inorganic material. In some particles the enmeshed bacteria gave the impression of having undergone some orderly arrangement inasmuch as they tended to lie with their long axes parallel. Other particles were found in which no bacteria could be demonstrated.

The composition of the bodies is thus in part organic and in part inorganic, a feature which they share with other biological structures such as shells, bones, pearls, calculi, etc. Indeed, it is not unreasonable to refer to these intra-colonial formations as " bacterial pearls " or " bacterial calculi." Further evidence that these bodies are biological formations rather than simple crystalline structures is provided by the observation that the morphology and tint of the bodies is to some extent characterised by the species of the associated organism. Fig. 4 shows the appearance of calcium carbonate crystals produced by the addition of ammonium carbonate solution to calcium chloride solution in the presence of serum. Fig. 5 shows carbonate bodies separated from a culture of *Bact. coli* ; these are angular and clearly distinguishable from the smooth, spherical bodies shown in fig. 6, which were separated from a culture of *Ps. pyocyanea.* This difference is not a function of size. It may be mentioned here that colonies of some strains of *Ps. pyocyanea* containing carbonate bodies present a very

BACTERIAL CALCULI
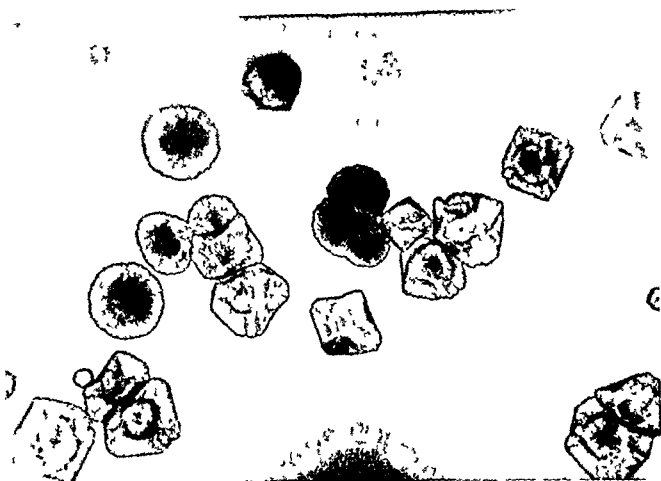
FIG 3 —Intra colonial carbonate bodies   × 80
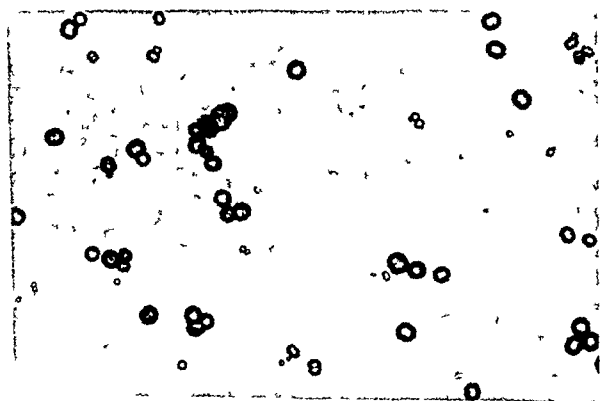
FIG 4 —Simple calcium carbonate precipitate   × 280

beautiful picture when viewed by polarised light, owing to the presence of both optically active carbonate bodies and optically active pyocyanin crystals.

Mode of formation of carbonate bodies

A probable explanation of the formation of calcium carbonate by micro-organisms is that calcium ions adsorbed on the bacteria react with ammonium carbonate secreted by the organism in the course of nitrogen metabolism. Such an explanation is in complete accord with the conditions found to affect the formation of intra-colonial carbonate bodies. In the interval between two closely adjacent colonies growing on a medium containing a high concentration of supplementary calcium, bodies resembling the intra-colonial formations may appear in the medium, showing that the reacting bacterial product is diffusible. Incorporation of sub-inhibitory concentrations of urea in the medium enhances carbonate formation by urease-producing organisms but not by non-producers of urease. The presence of a fermentable carbohydrate entirely suppresses carbonate production. This may be due to the formation of soluble calcium lactate, but it may equally well be due to inhibition of ammonification following the preferential use of the carbohydrate as an energy source (Kendall, 1922). Finally, when ammonium carbonate solution is allowed to diffuse from a central cup into a medium containing supplementary calcium, carbonate bodies resembling intra-colonial formations appear in the agar surrounding the cup.

The intimate combination of bacteria and carbonate found in the intra-colonial bodies might be explained by the fact that calcium ions are readily adsorbed on bacterial cells. McCalla (1940) reports that about 50 milli-equivalents of adsorbed magnesium per 100 g. of bacteria are replaceable by calcium, and Fulmer et al. (1921) report a reduction in the ash content of yeast from 6·3 to 3·0 per cent. as the result of growth in a medium free from magnesium and calcium. Thus the adsorbed calcium might be expected to react with the excreted ammonium carbonate at the surface of the bacterium, enclosing it within a carbonate shell. The focal distribution of the carbonate bodies within a colony is, however, more difficult to explain.

Resemblance of carbonate bodies to daughter colonies

Intra-colonial carbonate bodies are unlikely to be mistaken for the more commonly encountered appearances due to bacterial dissociation, such as daughter colony formation by mutants of *Bact. coli*. Other types of daughter colony have, however, been described (Hadley, 1927), which are extremely rhizoid and closely resemble crystalline deposits in the agar. Some writers state that such hard daughter colonies may be distinguished from crystalline deposits by crushing on a slide and staining. Clearly, such a manœuvre would fail to distinguish

daughter colonies from carbonate bodies, since the latter contain bacteria.

Cruickshank (1935) describes the appearance of clumps ("bodies or spots") in colonies of *Bact. typhi-flavum*, and confirms the observation by Hirsch (1934) of striking biconvex bodies ("Wetzsteinformen") which may appear in the centres of colonies of this organism growing on nutrient agar. No investigation of the chemical nature of these "wetzsteinformen" appears to have been made.

Carbonate bodies are readily distinguished from all forms of bacterial dissociation by examining the affected colonies by polarised light : carbonate bodies show distinct optical activity.

Calcium carbonate in relation to calculus formation

It has already been suggested that the complex of organic and inorganic material composing the carbonate bodies entitles them to be termed "bacterial calculi". It is interesting to consider whether such bodies may act as nuclei in the formation of calculi in man and animals. The high correlation between stasis, infection and calculus formation is well known. Eisenstaedt (1931), for example, reports that the nuclei of an entire series of 55 renal stones of various kinds examined contained bacteria which appeared to be material factors in their production. The same writer states that the likelihood of stone formation is markedly increased when the infecting organism is a urea splitter, and quotes Rovsing's (1923) opinion that a urea-splitting staphylococcus is the most frequent cause of recurrent renal lithiasis. In considering infection as a factor in calculus formation, attention must be paid to bacteriological rather than clinical infection : stasis and saprophytic infection may exist in semi-static animal secretions without inflammation. The emphasis on urea-splitting in relation to calculus formation suggests that the formation of calcium carbonate may be an important factor in the initiation of a calculus. It is the initiation of a calculus which poses the essential problem of lithiasis. Aggregations of crystalloid substances normally present in the animal secretions are capable of solution by dilution or by change of reaction, and purely protein aggregations are normally dispersable by proteolysis. Microbial carbonate calculi such as are here described cannot be dissolved by these processes, since they contain a substance not normally found in animal secretions. It has been observed that bacterial carbonate bodies will form in fluid cultures containing supplementary calcium as well as on solid media, and that in both cases a proportion of the particles are resistant to solution by $N/10$ hydrochloric acid.

Calcium carbonate is rarely the predominant constituent of a calculus except in the case of salivary, pancreatic, and prostatic calculi. A very high proportion of calculi do however evolve a few bubbles of gas with dilute hydrochloric acid. Such small quantities

BACTERIAL "CALCULI"
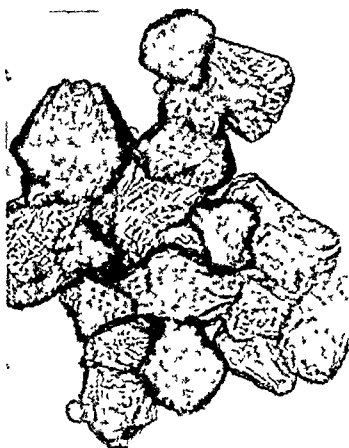
FIG 5—Angular carbonate bodies from a culture
of *Bact coli*   ×240

FIG 6—Globular carbonate bodies
from a culture of *Ps pyocyanea*
×240
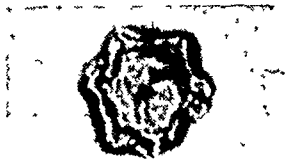
FIG 7—Calcium carbonate concretion
observed in the gall bladder bile of a
patient with mild cholecystitis and
many large gall stones   The bile was
sterile   ×450   (Rous Drury and
McMaster 1924 , fig 20)
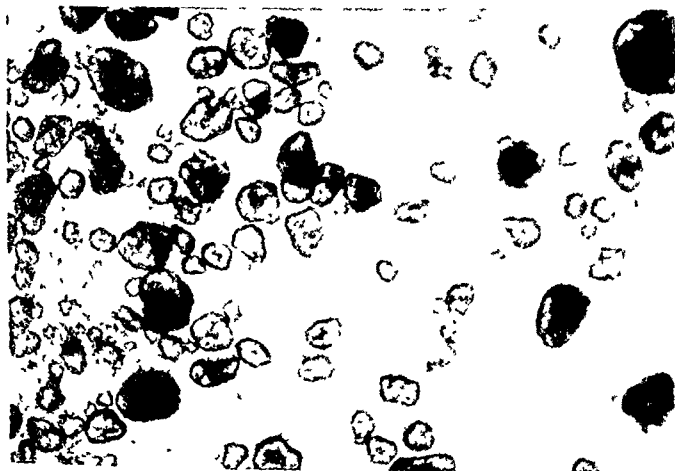
FIG 8—Nuclei deposited in the cannula of an intubated dog   ×250   (Rous,
McMaster and Drury 1924 , fig 11)

of carbonate can be appreciated if the powdered calculus is extracted with chloroform and water, and the deposit treated with acid while under observation with the microscope. It would be of considerable interest to know whether this very constant carbonate fraction resides in the nuclei of calculi.

It may be objected that the concentration of calcium present in animal secretions is far below the minimum concentration required in bacteriological media before visible carbonate bodies are produced. It must be remembered however that saprophytic organisms present in the ducts and receptacles of animal secretory organs are provided with a flowing medium and therefore with a continual supply of calcium. Furthermore such organisms, when colonised, are able to effect high local concentrations of calcium by their faculty of adsorption.

The production of calcium carbonate by bacteria growing in animal secretions is analogous to the production of calcium phosphate in similar situations by phosphatase-producing organisms (Glock *et al.*, 1938). The production of salivary calculi (Söderlund, 1920-21) and of dental tartar has been ascribed to such bacterial activity.

In the reports of Rous and his colleagues (Drury, McMaster and Rous, 1924; Rous, Drury and McMaster, 1924; Rous, McMaster and Drury, 1924) on experimental investigations relating to stone formation there appear numerous observations which support the hypothesis that calcium carbonate may play an important part in the provision of calculus nuclei. Stones which had formed in dogs intubated for the collection of bile under sterile conditions and in the absence of stasis consisted almost entirely of two substances—calcium carbonate and calcium bilirubinate. The carbonate calculi reacted to treatment with dilute acid in precisely the same manner as the bacterial calculi here described, an organic " shadow " remaining behind after the optical activity had been discharged by acid. Rous and his co-workers describe carbonate inclusions which they found at the centre of small human gall-stones, and carbonate " spheroliths " which they found in the gall-bladder sediment of cases of cholelithiasis and cholecystitis. Groups of these carbonate calculi are reproduced (figs. 7 and 8) from the papers by Rous and his colleagues (Rous, Drury and McMaster, fig. 7; Rous, McMaster and Drury, fig. 8). The resemblance to " bacterial calculi " is striking. Rous reports that the carbonate nuclei found in sterile dog bile were invariably associated with clumps of organic debris, and since the nuclei always appeared to develop within the organic material, he presumed that the formation of the carbonate depended upon some special condition existing within the organic material. It may be conjectured from the observations here reported concerning the mode of formation of " bacterial calculi ", that the special condition postulated by Rous to explain carbonate production within organic debris may have been the liberation of ammonium carbonate by autolysis of the organic material.

. It is reasonable to associate the findings of Rous and his colleagues with those described in this paper, by suggesting that "carbonate bodies" may be formed in various biological systems as the result of the local production of ammonium carbonate in the presence of soluble calcium. The ammonium carbonate may be formed by the autolysis of organic material or as a product of bacterial metabolism. and both sources may play a part in the formation of calculus nuclei.

Summary

The formation within colonies of various bacterial species of optically active bodies composed largely of calcium carbonate is described.

These bodies appeared in cultures growing on ordinary routine media and the phenomenon is associated with an unusually high calcium content in the medium.

Evidence is presented to show that these bodies are biological structures having an organic and an inorganic part, and the term " bacterial calculi " is therefore suggested.

The mode of formation of the bodies is discussed.

Attention is drawn to the possibility of confusing the carbonate bodies with manifestations of bacterial dissociation.

The significance of the carbonate bodies in relation to calculus formation in man and animals is discussed.

REFERENCES

BUCHANAN, R. E., AND FULMER, E. I. — 1928. *Physiology and biochemistry of bacteria*, vol. i, p. 138.

" " " " — 1930. *Ibid.*, vol. iii, p. 216.

CRUICKSHANK, J. C. — 1935. *J. Hyg.*, Camb., xxxv, 354.

DRURY, D. R., McMASTER, P. D., AND ROUS, P. — 1924. *J. Exp. Med.*, xxxix, 403.

EISENSTAEDT, J. S. — 1931. *Surg. Gyn. Obst.*, liii, 730.

FULMER, E. I., NELSON, V. E., AND SHERWOOD, F. F. — 1921. *J. Amer. Chem. Soc.*, xliii, 191.

GLOCK, G. E., MURRAY, M. M., AND PINCUS, P. — 1938. *Biochem. J.*, xxxii, 2096.

HADLEY, P. — 1927. *J. Inf. Dis.*, xl, 1.

HIRSCH, W. — 1934. *Cbl. Bakt.*, Abt. I, Orig., cxxxi, 39.

KENDALL, A. I. — 1922. *J. Inf. Dis.*, xxx, 211.

McCALLA, T. M. — 1940. *J. Bact.*, xl, 23.

ROUS, P., DRURY, D. R., AND McMASTER, P. D. — 1924. *J. Exp. Med.*, xxxix, 97.

ROUS, P., McMASTER, P. D., AND DRURY, D. R. — 1924. *Ibid.*, xxxix, 77.

SÖDERLUND, G. — 1920-21. *Acta. chir. Scand.*, liii, 189.

WINSLOW, C.-E. A., AND HOTCHKISS, MARGARET — 1921-22. *Proc. Soc. Exp. Biol. and Med.*, xix, 314.

GROWTH AND REPAIR IN ADIPOSE TISSUE

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(PLATE CII)

MUCH uncertainty exists about the growth and regeneration of adipose tissue. So far as we know, no-one has submitted the problem to a quantitative study, although all pathologists are aware that fat increases or decreases in amount under varying conditions of metabolism and at various epochs of life. In this paper we describe some experiments designed to show whether adult fatty tissue regenerates under the stimulus of partial removal of the parent mass.

METHODS

We have worked with the bilateral folds of omentum-like tissue attached to the testes of rats. For convenience we refer to this as testicular omentum. Healthy male albino Wistar rats, 8 weeks old, were selected for uniformity in body weight. Under open ether anæsthesia and with strict aseptic precautions, the abdominal cavity was opened through a sub-umbilical mid-line incision and about half of one or both testicular omenta removed. Gentle traction on the testis was necessary for this manœuvre, but no permanent damage of that organ resulted in any of the animals. The stump of the omentum was ligatured with cotton thread ; in a few animals no ligature was used, bleeding being controlled with a cautery. The abdomen was closed with two layers of continuous cotton sutures. Little bleeding occurred and the animals made an uninterrupted recovery. Stitch abscesses developed in several animals but cleared up without much trouble after a few weeks. Four groups of rats were employed. In one group, about half of the left testicular omentum, in the second, half of both left and right testicular omenta was removed. In the third, a portion of the gastric omentum was detached, the amount of tissue being about half of that obtained from the testicular omentum because the gastric omentum is a scanty structure in 8-weeks-old rats. This group served as a control to the first and second groups, since all operative procedures were similar in the three categories, with the exception of the type of tissue removed. A fourth series, killed at the time of operation, provided data on the normal weights of the structures concerned. The table shows a striking similarity in body weights between all 4 groups, so that we feel justified in applying the mean values for the testicular omenta obtained from group 4 to the other groups in assessing repair of the removed adipose tissue.

In addition, we removed testicular omentum from a further group of 8-weeks-old rats for the purpose of studying the histological changes in the stump at varying times after resection. Finally, autologous grafting of the testicular

omentum was performed in 10 three-weeks-old rats, the graft being attached to the peritoneum by a single cotton thread.

The duration of the resection experiments was three months and during this time the rats were given unlimited amounts of M.R.C. rat cubes and water. No more than 5 animals lived in the same cage. Repeated weighing indicated a steady increase in growth once the set-back consequent on the operation was overcome. At the end of the experimental period the rats were quickly killed with ether and chloroform, their testes and omenta carefully dissected out, separated, lightly swabbed with cotton wool and weighed. Material for microscopic examination was fixed in 10 per cent. formol-saline and frozen and paraffin sections prepared in the usual fashion for staining with Sudan III, Ehrlich's acid hæmatoxylin and eosin and Weigert's iron hæmatoxylin and van Gieson. A few animals were injected through the abdominal aorta with a suspension of india ink prior to making spreads on a glass slide of the whole omentum, the object of this technique being to obtain a clearer idea of the distribution of the blood vessels and the lobules of fat in the intact organ. This device is of especial value in studying the omentum in young rats.

RESULTS

The normal testicular omentum

At birth, the normal testicular omentum of the albino rat is no more than a minute tubular peritoneal fold which is found with some difficulty. It consists of small clumps of rounded or polyhedral cells
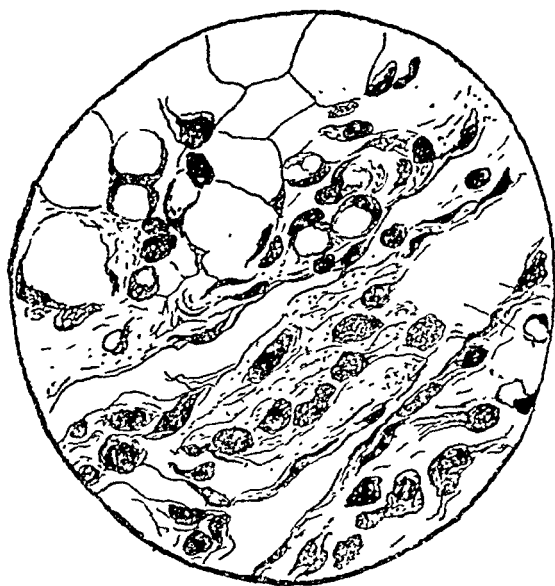
FIG. 1.—Spread of testicular omentum from 7-days-old rat showing all stages of developing fat cells. Camera lucida drawing. ×400.

grouped together in close relationship to a vascular tree derived as an offshoot from the main vessels supplying the testis. Fat granules or small globules are found within these cells, few of which resemble the
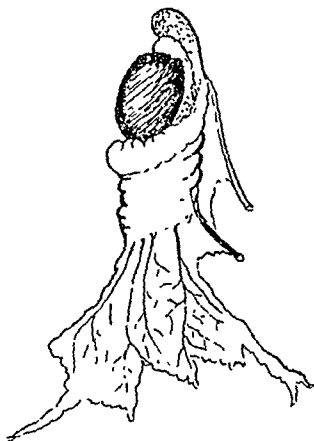
Fig. 2.—Mature testicular omentum attached to testis and epididymis.   ×2 (c).
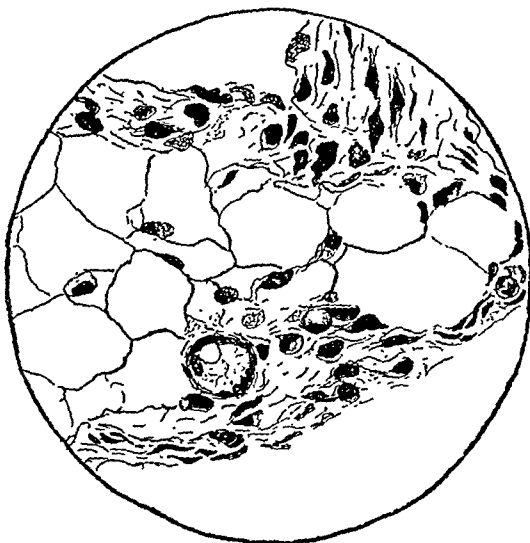
Fig. 3.—Spread of testicular omentum from 17-days-old rat, showing small islands of
primitive fat cells, one close to a vessel.   Camera lucida drawing.   ×400.

signet cells of mature adipose tissue. Some are quite free from fat inclusions, seeming to exist as a sheath around the smaller vessels. The organ possesses a simple leaf-like structure whose base is apposed to the capsule enclosing the testis. By three weeks after birth the omentum is several mm. long and shows more complicated branching.

(a)   (b)

(c)   (d)
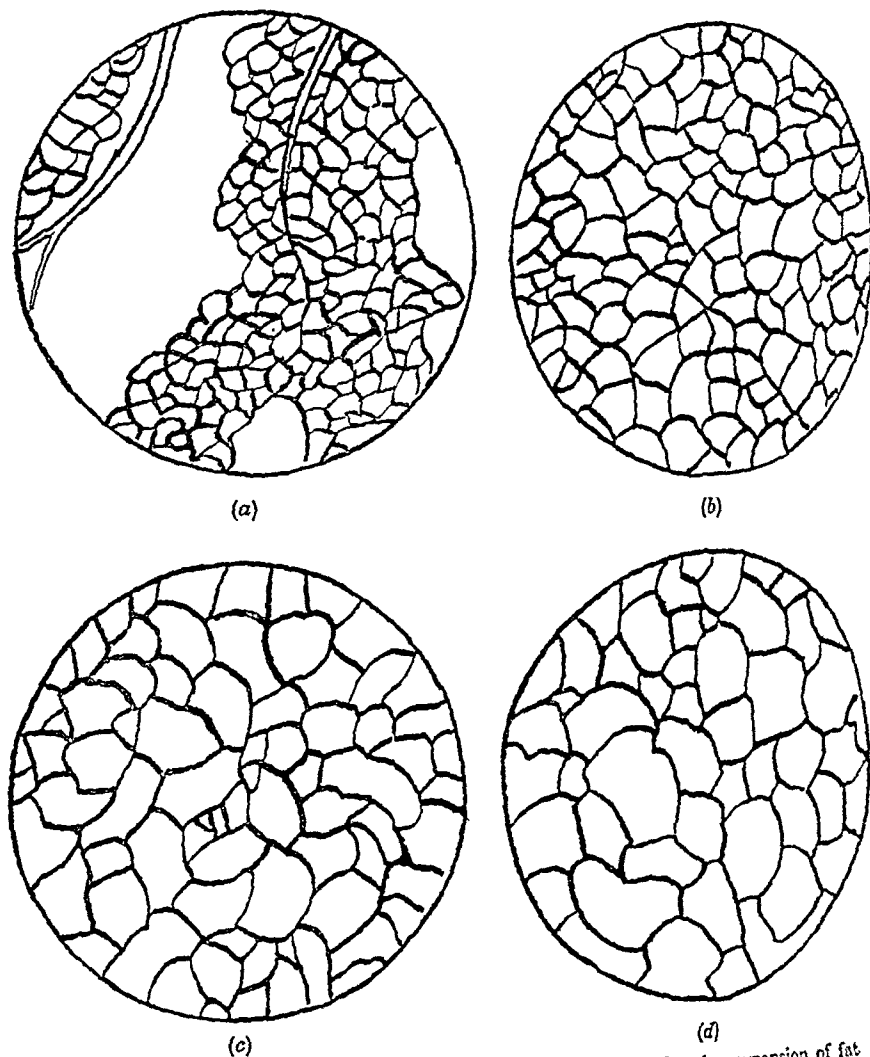
FIG. 4.—Projection drawings of testicular omental spreads showing expansion of fat cells with increasing age : (a) 7 days, (b) 23 days, (c) 56 days, (d) 128 days. ×150.

whilst white fat is plainly seen in its substance. Fat cells are small, though many are filling up with fat, and all stages from a few granules through foamy cytoplasm to coarse globules exist side by side (fig. 1). During the first and second months of life growth proceeds at a good pace, chiefly through elongation and branching of the organ (fig. 2). The fat cells become larger and soon reach the adult stage. Perivascular sheaths of small cells containing tiny fat granules can be found

without much difficulty (fig. 3). The fatty tissue now takes on the appearance of tiny discrete masses separated by thin folds of peritoneum associated with the ramifying blood vessels. A fine capillary plexus penetrates each mass and maintains an intimate connection with every fat cell. It does not seem to be appreciated generally how vascular adipose tissue is, though this fact has been emphasised recently by Gersh and Still (1945) and can be confirmed quite easily by injection of the main vascular supply of the structure. Expansion of fat cells by fat storage proceeds well into maturity in the rat (fig. 4) and without doubt is largely responsible for omental growth. Dr J. D. Judah kindly estimated the fat content of a few mature omenta and found that as much as 90 per cent. of the dried weight was fat. Nevertheless, an increase in number of cells plays a part, the elongation of the main vascular trunks being accompanied by new formation of minute fat-cell clumps along their distal expansions. Close scrutiny fails to disclose any evidence of division of pre-existing fat cells and it is difficult to avoid the impression that growth is the result of differentiation of cells initially free from fat in close association with the growing blood vessels. Speed of growth is considerable in the white rat, and reference to our table indicates that the right testicular omentum may enlarge from 0·22 g. at 2 months to 1·24 g. at 5 months, a sixfold increase. During this period body weight just about doubles itself, so that the omental expansion seems to be something more than just part of a general somatic phenomenon. Our table also brings out the remarkable agreement in behaviour of the two omenta.

Fat grafts

Like Hausberger (1938) we have been able to demonstrate the growth of autologous fatty grafts provided with a favourable nidus in the peritoneum. Hausberger transplanted the omental rudiment shortly after birth and obtained large masses of adipose tissue, resembling the intact organ in size and structure. Unfortunately he gives no details from which to assess the success of his experiments. We used tissue from somewhat older animals (three weeks after birth) and were less successful than Hausberger appears to have been. In no instance did we meet with a graft which approached the size of the omentum corresponding to the age of the animal, but technical faults may have been responsible. However, we did not pursue the matter very far. We found no evidence of direct multiplication of fat cells in the graft and concluded that expansion of the tissue follows the normal plan of growth.

Quantitative changes after partial removal of the testicular omentum

Our results are summarised in the table. Group 4 of 8-weeks-old rats may be taken as an indicator of conditions holding for group 1

at the time of operation.   The left testicular omentum averaged 0·22 g.
for a body weight of 116 g.   An average amount of 0·12 g. was
removed from each rat leaving, we may assume, 0·10 g. of left
testicular omental tissue behind.   After 3 months this fragment
increased to a mean value of 0·46 g.   Control rats (group 2) of about
the same mean body weight (120 g.) increased their left omental tissue
from an assumed mean of 0·22 g. to 1·24 g.   In other words, the
resected animals grew an average amount of 0·36 g. of fat in three
months, whereas the controls produced 1·02 g., nearly three times as
much.   Obviously there was no attempt to make good the deficit in
the resected rats, which suggests that partial removal of the omentum

TABLE

*Body weight and weight of testicular and gastric omenta before and
after partial removal of omentum*

Group	No. of rats in group	Mean body weight at operation (g.)	Mean weight of tissue removed from testicular or gastric omentum (g.)	Mean body weight 3 months after operation (g.)	Mean weight of testicular omentum (g.)			
					at operation		3 months after operation	
					Right	Left	Right	Left
1. Removal of part of left testicular omentum from rats 8 weeks old	15	117	0·12	222	...	...	1·08	0·46
2. Removal of part of gastric omentum from rats 8 weeks old	15	120	0·06	228	...	...	1·24	1·24
3. Removal of part of both testicular omenta from rats 8 weeks old	9	106	0·29 (R+L)	215	...	...	0·34	0·23
4. Normal rats 8 weeks old	16	116	...	...	0·22	0·22	...	...

does not act as a local stimulus for growth.   The speed of growth in
the two groups seems to have been much the same, for the resected
animals increased their left omental tissue 4·58 times (from 0·10
to 0·46 g.), the controls 5·55 times (from 0·22 to 1·24 g.) over the
same period of time.   This again indicates contempt for the local
stimulus.   Our data give no reason for thinking that the intact right
testicular omentum compensates in any way for its depleted fellow,
since apparently it grew more or less at the expected rate.   (The mean
of 1·08 g. for the right testicular omentum is certainly less than that
of 1·24 g. for the control group, despite the close agreement in respective
body weights—222 and 228 g.—but the discrepancy is probably one
of sampling.   If compensation had occurred the right omentum
would have been much heavier than in the control group.)   There
may have been compensation for this local loss elsewhere in the
body fat, since the stores of adipose tissue are considerable, but this

assumes a unity of function throughout the fatty mass of the organism and homeostasis in the system. We possess no information about such matters; on the contrary, pathological experience suggests a fair degree of isolationism amongst the adipose tissues of the body.

The experiments in which both omenta were partly resected also gave no evidence of regeneration, but the number of animals employed was too few to warrant much attention. We conclude from these results that adipose tissue shows little or no local regeneration under the stimulus of partial removal, even though it be growing actively.

Microscopical changes at the site of partial removal of testicular omentum

Close study of the region adjacent to a line of resection of testicular omentum has failed to convince us of the occurrence of repair of mature fat cells. Our preparations cover a period of 6 months.

Twenty-four hours after resection the capillaries in the fatty tissue close to the line of section appear very numerous and are distended with red corpuscles. Migration of leucocytes from the small vessels is going on and these cells can be traced to the divided surface, where they are packed together in a fairly wide zone together with a few lymphocytes and macrophages, some strands of fibrin and remnants of divided fat cells. Leucocytes also extend a short distance between the deeper normal fat cells. Macrophages are most numerous where fat cells have ruptured and show great variation in size and shape. Some are elongated and star-shaped, resembling young fibroblasts, and these appear to be formed in the vicinity of capillaries. A few contain tiny globules of fat. The mature fat cells around the incision are normal.

After 2 days the divided omentum is swollen and much congested, due to dilatation and opening up of most of the capillaries. Along the cut edge there is dense infiltration with mononuclear cells, fewer leucocytes and a certain number of large cells like fibroblasts arranged at right angles to the general direction of the fat cells and in close relation to capillaries. A few "foam" cells are present, and some mature fat cells showing shrinkage and vacuolated fat, but there is no evidence of division of these cells.

By 4 days a wide zone of reparative tissue exists at the cut edge, composed of mononuclear cells, large lymphocytes, plasma cells and fibroblasts. Fibrillation is going on actively near the necrotic remnants and new capillaries grow in from the deeper capillary plexuses around the mature fat cells. Around these, also, are many inflammatory cells, some of which invade the occasional degenerate fat cells found here. Huge macrophages stippled with fat occur wherever fat cells are shrunken and breaking up. A few large spaces filled with fat are outlined by rings of elongated mononucleated cells, suggesting the encirclement of effused fat with macrophages. These are the earliest indications of future oil cysts enclosed by giant cells. There is still

no evidence of division of fat cells, though it would be easy to mistake some of the large fat-containing macrophages for young fat cells.

At 5 days a well defined reparative zone covers the divided surface of the omentum and much of the deeper capillary congestion has disappeared, although the increased cellularity of this region still persists. Fibroblasts in all stages of development are aligned parallel to the surface and are associated with numerous fine fibrils staining light pink or yellow with van Gieson. Some of these are reticulum fibres. Small collections of leucocytes and lymphocytes persist near the surface or around fat-cell remnants. The mature fat cells are sharply separated from the granulation tissue and present no convincing evidence of cell division. A few of them are very large and contain foamy protoplasm and very large nuclei but no mitotic figures. Small oil cysts are forming, surrounded by foam cells.

At 7 days collagen and reticulum are well in evidence around fibroblasts, foam cells are numerous and mitotic division is going on in some of the mononuclear cells, but mature fat cells are not proliferating. Foreign-body giant cells now appear close to the ligature and small oil cysts are forming.

By 14 days there is complete covering of the divided surface with organising fibrous tissue rich in mononuclear cells and fibroblasts. Strands of this tissue penetrate for some distance into the omentum between the fat cells. Concentric whorls of young fibrous tissue and newly formed giant cells enclose ligature remnants and small oil cysts or massive cholesterol crystals (fig. 5). Foam cells are still numerous, some quite large, with a lace-like cytoplasm, but none of the mature fat cells show signs of division.

From now on there is progressive fibrosis and giant-cell formation around the ligature remnants and organisation of the reparative tissue over the cut surface. A few oil cysts persist for long periods enclosed in giant cells and whorls of fine fibrous tissue (fig. 6). At no time throughout the six months after operation is there evidence of proliferation of fat cells. In the later months the divided omental surface becomes smooth and thin and is occasionally attached to abdominal structures by fibrous adhesions.

We conclude from our microscopical studies that division of the testicular omentum leads to *aseptic inflammation and repair by granulation and fibrous tissue*. Proliferation of mature fatty cells does not occur, either in the zone of tissue adjacent to the line of injury or at a distance from it.

DISCUSSION

Discussion of regeneration in adipose tissue requires that we rid our minds of the view that this tissue is a purely inert storage depot with little metabolism of its own. The modern investigations of Schoenheimer and his associates (Schoenheimer, 1942) have demon-

GROWTH AND REPAIR IN ADIPOSE TISSUE
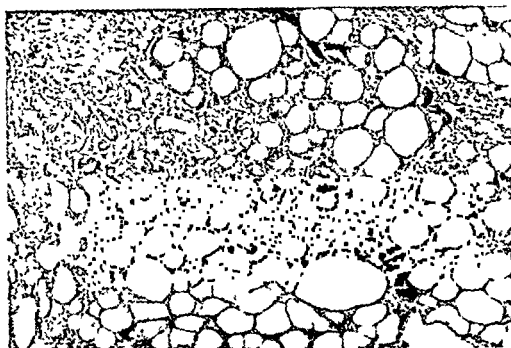
FIG 5 —Testicular omentum 14 days after removal of about half the organ   Organising granulation tissue, foam cells and giant cells grouped around damaged fat cells   H and E   × 100
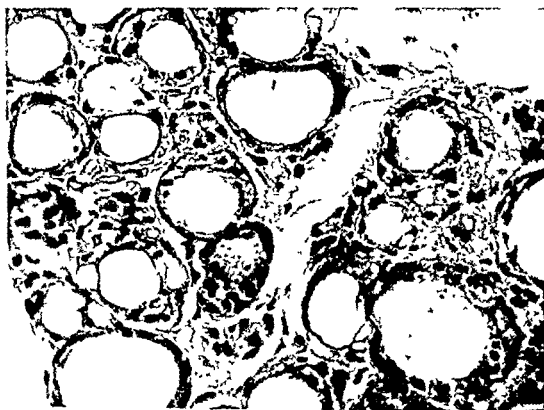
FIG 6 —Foreign body giant cells enclosing small oil cysts in testicular omentum three months after partial resection of the organ   H and E   × 450.

strated the state of continuous flux in which fat cells exist. A copious blood supply with potentialities scarcely inferior to those of some forms of muscle (Catchpole and Gersh, 1947), provides for a not inconsiderable oxygen consumption (Hoffmann and Wertheimer, 1927) ; indeed, there are varieties of fatty tissue, the so-called brown fat, with a respiration as great as that of the kidney (Fleischmann, 1929) and a rich complement of enzyme systems such as succinate and pyruvate oxidases, cytochrome oxidase, ascorbic acid and diphosphothiamine and lipases (Hook and Barron, 1941 ; Fawcett, 1947). Adipose tissue, therefore, should be looked upon as a highly specialised, well differentiated organ rather than a modification of connective tissue, although no doubt it shares some of the functions of the latter.

For this reason it is not surprising that regeneration is an inconspicuous feature of fat cells, for a good deal of biological observation supports the generalisation that highly differentiated tissues are often poor at regeneration.

The literature is full of uncertainty on these matters and at least two sets of opinions have been expressed about normal growth of white fat.

1. Many writers agree that it is a derivative of connective tissue. Flemming (1871 *a* and *b*, 1876) stated that any such cells, especially when young and in close relation to blood vessels, can be converted into fat cells. He postulated slowing of the blood flow in dilated blood vessels with increased transudation into connective tissue as important factors in the transformation. " Das Fettgewebe ist eine physiologische Form des Bindegewebes ". von Kölliker (1886, 1889-1902) believed that fat droplets are deposited in connective tissue cells and support for this view has been adduced by Hammar (1895), Bell (1909), Berg (1910-11) and Foot (1912). Chiari (1909-12) distinguished two sets of fatty tissue cells, both derived from primitive mesenchyme, and Inglis (1926-27) agreed that white fat is a variety of connective tissue, though the glandular brown fat of the axillæ, mediastinum and subpleural regions bears some relation to lymph and hæmolymph nodes.

2. Some writers hold that a special stem cell is responsible. Toldt (1870) described specific anlagen in the embryo with characteristic vascular systems and lobular groupings of fat cells, and Mallory (1914) agreed that a special fat cell is set apart early in development for fat storage. Wassermann (1925-26) refers the embryonic origin of all fatty tissues to primitive fat organs which are clumps of branched or even syncytial cells associated with numerous capillaries and very little connective tissue. Hæmopoiesis goes on in these organs, which have much in common with bone marrow. Wassermann believes that primitive fat is akin to reticulo-endothelial tissue arising from the mesenchyme of vessels. Recent work by Tedeschi (1946) supports this view. Storage of vital dyes by embryonic fat cells and to a less extent by mature fat cells has been described by Volterra (1923), Dogliotti

(1928-29) and Bremer (1937-38), observations which give some support to the reticulo-endothelial theory.

No decision about regeneration of fat cells has been reached. Division of pre-existing fat cells was described by Flemming (1871 a and b), Pfeifer (1892), Rothmann (1894), Schujeninoff (1897), Cornil and Ranvier (1901) and Marchand (1901) in a variety of pathological conditions. Rehn and his co-workers (Rehn, 1912, 1913; Eden and Rehn, 1914; Eden, 1917) and Eisleb (1916) followed the behaviour of autologous and homologous fatty grafts in animals and man, emphasising the production of young fat cells from pre-existing mature cells by amitosis. Rehn at no time saw mitotic division in adipose cells. He gave an accurate account of aseptic inflammation and the formation of oil cysts and giant cells, but his description of young fat cells carries no conviction, for it fails to differentiate them from macrophages which have ingested fat granules. Marchand (1920), though well aware of the difficulties introduced by phagocytosis of fat, accepted the evidence in favour of fat-cell regeneration. He made the curious statement that the membrane of degenerating fat cells behaves like collagen fibres in connective tissue, and new cells arise from that portion of the protoplasm which contains the nucleus. All workers with fatty grafts admit, however, that true regeneration is slight.

In an investigation of experimental osteomyelitis, Enderlen (1899) described giant-cell production and formation of fat cells around the injured area between the 8th and 16th days. He derived the new fat cells from proliferating connective tissue. Hayashi (1915) reached somewhat similar conclusions from an experimental study of fat necrosis in subcutaneous tissue, though he placed more emphasis on histiocytes than on fibrocytes. A straightforward denial of fat regeneration has been given by Maximow (1903-04, 1912), Ziegler (1904), Marx (1910) and Makkas (1912), based on experimental studies in which the part played by macrophages has been given proper recognition. von Verebély (1907) accepted an origin from mono-nuclear phagocytes and mature fat cells.

Our investigations give no support to the idea of regeneration of adipose tissue, since neither grafts nor traumatised omental white fat showed proliferation of fat cells, although repair by fibrous tissue production went on in the accustomed fashion in both. Our quantitative studies afford convincing proof that a local stimulus of partial resection does not stimulate compensatory hypertrophy in a fat organ. Nevertheless, such a tissue as the testicular omentum grows vigorously at a speed several times that exhibited by the organism as a whole. The explanation of this paradox is not clear, though it seems likely that the growth of fat is incremental rather than proliferative, in the sense that new fat cells are produced through differentiation of precursors in close association with an expanding vascular framework.

SUMMARY

A study of growth and repair in adipose tissue such as the testicular omentum of the albino rat leads to the conclusion that mature fat cells possess no proliferative capacity, although new fatty tissue can be laid down by modification of cells in close association with blood vessels.

REFERENCES

BELL, E. T. . . . . 1909. *Amer. J. Anat.*, ix, 401.

BERG, W. . . . . 1910-11. *Z. Morph. Anthropol.*, xiii, 305.

BREMER, J. L. . . . . . . 1937-38. *Anat. Rec.*, lxx, 263.

CATCHPOLE, H. R., AND GERSH, I. 1947. *Physiol. Rev.*, xxvii, 360.

CHIARI, H. . . . . 1909-12. *Trans. Chicago Path. Soc.*, viii, 65.

CORNIL, A. V., AND RANVIER, L. . 1901. Manuel d'histologie pathologique, 3rd ed., *Paris*, vol. i, p. 154.

DOGLIOTTI, G. C. . . . . . 1928-29. *Z. Zellforsch.*, viii, 222.

EDEN, R. . . . . 1917. *Dtsch. med. Wschr.*, xliii, 424.

EDEN, R., AND REHN, E. . . . 1914. *Arch. klin. Chir.*, civ, 65.

EISLEB, H. . . . . 1916. *Beitr. klin. Chir.*, cii, 249.

ENDERLEN . . . . 1899. *Dtsch. Z. Chir.*, lii, 293.

FAWCETT, D. W. . . . . . 1947. *Science*, cv, 123.

FLEISCHMANN, W. . . . . . 1929. *Arch. ges. Physiol.*, ccxxii, 541.

FLEMMING, W. . . . . . . 1871a. *Arch. mikr. Anat.*, vii, 32, 328.

    „   . . . . 1871b. *Arch. path. Anat.*, lii, 568.

    „   . . . . 1876. *Arch. mikr. Anat.*, xii, 434.

FOOT, N. C. . . . . 1912. *Beitr. path. Anat.*, liii, 446.

GERSH, I., AND STILL, M. A. . . 1945. *J. Exp. Med.*, lxxxi, 219.

HAMMAR, J. A. . . . . . . 1895. *Arch. mikr. Anat.*, xlv, 512.

HAUSBERGER, F. X. . . . . 1938. *Arch. path. Anat.*, cccii, 640.

HAYASHI, I. . . . . 1915. *Frankf. Z. Path.*, xvii, 72.

HOFFMANN, A., AND WERTHEIMER, E. 1927. *Arch. ges. Physiol.*, ccxvii, 728.

HOOK, W. E., AND BARRON, E. S. G. 1941. *Amer. J. Physiol.*, cxxxiii, 56.

INGLIS, K. . . . . 1926-27. *J. Anat.*, lxi, 452.

VON KOLLIKER, A. . . . . . 1886. *Anat. Anzeig.*, i, 206.

    „   . . . . 1889-1902. Handbuch der Gewebelehre des Menschen, 6th ed., *Leipzig*.

MARKAS, M. . . . . 1912. *Beitr. klin. Chir.*, lxxvii, 523.

MALLORY, F. B. . . . . . 1914. The principles of pathologic histology, *Philadelphia* and *London*, p. 301.

MARCHAND, F. . . . . . . 1901. Der Prozess der Wundheilung, *Stuttgart*, pp. 137 and 219.

    „   . . . . 1920. *Beitr. path. Anat.*, lxvi, 1.

MARX, G. . . . . 1910. *Z. Ohrenheilk.*, lxi, 7.

MAXIMOW, A. . . . . . . 1903-04. *Beitr. path. Anat.*, xxxv, 93.

    „   . . . . 1912. *Arch. Entwicklungs-Mech.*, xxxv, 135.

PFEIFER, V. . . . .   1892.   *Dtsch. Arch. klin. Med.,* l? 438.

REHN, E.   . . . .   1912.   *Arch. klin. Chir.,* xcviii, 1.

,, ...   1913.   *Ibid.,* ci, 962.

ROTHMANN, M.   . . . . .   1894.   *Arch. path. Anat.,* cxxxvi, 159.

SCHOENHEIMER, R. . . . : . .   1942.   The dynamic state of body con-
stituents. *London* and *Cambridge,
Mass.,* pp. 21 and 23.

SCHUJENINOFF . . . . . .   1897.   *Beitr. path. Anat.,* xxi, 1.

TEDESCHI, C. G.   . . . . .   1946.   *Arch. Path.,* xlii, 320.

TOLDT, C.   . . . .   1870.   *Sitzungsber.   Wien. Akad. Wien
Math.-Naturwiss.,* lxii, Abt. II, 1.

VON VEREBÉLY, T. . . . . .   1907.   *Beitr. klin. Chir.,* liv, 320.

VOLTERRA, M. . . . . . .   1923.   *Sperimentale,* lxxvii, 242.

WASSERMANN, F.   . . . . .   1925-26. *Z. Zellforsch.,* iii, 235.

ZIEGLER, K.   . . . . . .   1904.   *Beitr. path. Anat.,* xxxvi, 435.

SHORT ARTICLES

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TUBERCULOSIS OF THE BRAIN AND OVARY IN A BIRD

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Tuberculosis is a relatively common disease of domestic poultry (e g. Gallagher, 1921, Feldman, 1938) and has often been recorded in wild birds in captivity, although it is doubtful if it occurs in the wild state (Fox, 1923, Scott, 1930, Hamerton, 1935) Most birds appear to be susceptible only to the avian type (F Griffith, 1911, p 171), although parrots are prone to cutaneous lesions due to the human type and infection may spread from parrot to man and vice versa (Scott) Human infection with the avian strain is probably rare (Branch, 1931)

Among wild birds in captivity, the organs most commonly affected would appear to be the liver, spleen, lungs and air sacs in order of descending frequency, although the exact order differs among authorities In all series the liver heads the list No case with involvement of the ovaries is recorded by Fox, but Scott saw one, possibly due to direct spread from infected kidneys However, Gallagher in domestic fowls and Hamerton in wild birds found ovarian lesions not infrequently

Scott found one tubercle in the meninges of an Australian rail, *Rallus pectoralis* Feldman could find no reference to a lesion of the brain itself in any bird In his own series the brains were submitted to microscopical examination with negative results, but others have not been as thorough

The following case, therefore, exhibits a very unusual distribution of lesions, that in the brain being possibly unique

Case report

The subject was a female mandarin duck, *Aix galericulata*, which had lived in the Zoological Gardens in Regent s Park for four years and two months All this time it had been in an open enclosure with other water fowl among whom cases of tuberculosis had occurred sporadically Its exact age and origin were not known, since it had been presented by a private collector It died unexpectedly

At necropsy the bird was found to be moderately well nourished and in good plumage The air passages and air sacs were normal, but the lungs contained many caseous nodules up to 1 cm in diameter In the kidneys, tuberculous masses were numerous and reached a diameter of 2 cm A number of minute tubercles were seen in the ovaries, which were in the state of winter quiescence and contained no mature ova

The peritoneal cavity contained much turbid fluid, as is usual in tubercle in birds, and there was a heavy deposit of greenish fibrin on all the serous surfaces, but no tubercles The parenchyma of the liver showed fatty change, but no tubercles were found on repeated section The spleen was enlarged and caseation replaced most of the parenchyma

Within the cranium the meninges appeared normal and the dura stripped readily from the leptomeninges which, with the surface of the brain, were of normal appearance. However, a soft hæmorrhagic nodule, about 3 mm. in diameter, lay some 2 mm. below the surface of the right cerebral hemisphere.

No abnormalities were found in any other of the organs.

Histological appearances

Spleen and kidneys. These organs showed typical avian tubercles of a rather chronic type, namely, a central area of necrosis, with a marginal zone of lymphocytes and fibroblasts. Innumerable acid-fast bacilli were present at the periphery of these lesions.

Ovary. Tubercle bacilli were seen within the smaller blood vessels and these formed the starting point of a number of early tubercles. It was evident that these organisms had not come from the kidney by direct spread. The tubercles lay very close to the developing ova.

Brain. The lesion found on naked-eye examination was a typical avian tuberculoma of a rather chronic type with fibrous wall. Innumerable acid-fast bacilli were present in all parts of the section examined, being found in masses in the small arteries round which tubercles were forming. In some places the organisms appeared to be caught up in thrombi within the vessels. Small tubercles surrounded the main lesion.

Discussion

In birds tuberculosis is thought to enter by the digestive tract as a rule and to spread by the blood stream. Bland Sutton (1886) saw the bacilli in clusters in the veins, noted the frequency of involvement of the liver, and advanced an ingenious theory to account for the spread by invoking mechanical pressure exerted by the swelling of this organ and of the spleen when their vessels were blocked by infected thrombi. In the present case the bacilli were evident within blood vessels, but these were usually arterioles, especially in the brain ; the liver was not involved.

In birds the lymphatic system is known to be well developed in such type species as geese and ducks (Lauth, 1824), fowls and doves (Josifoff, 1930). It consists of a number of large vessels which accompany the main blood vessels and open on each side into the angle between the jugular and subclavian veins. Lymph nodes do not appear to be described, so that lymphatic spread cannot be traced by their involvement. It is curious to note that involvement of the " cervical lymph glands " has been reported (F. Griffith). Histological details are lacking and these may have been subcutaneous nodules or may have developed in the lymphatic plexus which accompanies the jugular vein on each side (Josifoff). If so, their occurrence would seem to indicate lymphatic spread.

Danger to man from infected eggs used as food does not appear to be real. Fitch and Lubbehusen (1927-28), confirming earlier statements of Gallagher, found that tuberculous hens layed fewer eggs than usual, that only one per cent. of those surviving were infected and that most of these were infertile. Klimmer (1931), however, found as many as 3 per cent. of such eggs to be infected. Most authors do not consider man to be very susceptible to the avian bacillus (e.g. Branch, 1931), while A. S. Griffith (1928-29) in 54 cases of tubercle in other mammals found the avian strain in only two, both marsupials.

The rarity of involvement of the brain in birds is difficult to explain. In the classical experiments of F. Griffith a number of animals were infected with avian bacilli, but brain lesions are not mentioned as occurring in any. Possibly, therefore, absence of cerebral involvement is characteristic of the avian form of tuberculosis. If the normal method of spread is by the veins, the brain might

well escape infection on account of the negative pressure within its venous sinuses   Arterial spread would not be subject to this limitation and this is what has occurred in the present case

Summary

A case of tuberculosis in a mandarin duck (*Aix galericulata*) is described   Lesions occurred in the lungs, spleen, kidneys, ovary and brain   In birds, tuberculosis of the ovary is uncommon and in the brain it appears to be very rare   In birds, tubercle bacilli usually spread by invasion of the veins   In the brain of this duck, the arterioles were involved, which may explain the unusual site of the lesion   It would appear, however, that avian tubercle bacilli rarely involve the brain in experimental infections of other animals, even when lymphatic spread occurs

I have to thank the Zoological Society of London for permission to publish this case

REFERENCES

BRANCH, A	1931	*Arch Path* , xii, 253
FELDMAN, W H	1938	Avian tuberculosis infections, *London*, p 205
FITCH, C P , AND LUBBEHUSEN, R E	1927 28	*J Amer Vet Med Assoc* , lxxii, 636
FOX, H	1923	Disease in captive wild mammals and birds, *Philadelphia, London and Chicago*, p 484
GALLAGHER, B A	1921	*Farmers' Bull U.S Dept Agric* , no 1200
GRIFFITH, A S	1928 29	*J Hyg* , Camb , xxviii, 198
GRIFFITH, F	1911	Royal Commission on Tuberculosis, Final Report, Appendix, vol iv, p 149
HAMERTON, A E	1935	*Brit J Tub* , xxix, 145
JOSIFOFF, J M	1930	*Anat Anzeiger*, lxix, 213
KLIMMER, M	1931	*Munch med Wchsr* , lxxviii, 1212
LAUTH, E A	1824	*Ann des Sci Nat* , iii, 381
SCOTT, H H	1930	Medical Research Council, Spec. Rep Ser , *London*, no 149, p 217.
SUTTON, J BLAND	1886	*J Comp Med and Surg* , vii, 329

NATURAL AND ACQUIRED IMMUNITY IN FROGS AND FISH

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In two previous papers (Bisset, 1946, 1947a) I have described the effect of temperature upon the host parasite relationship in bacterial infections of cold blooded vertebrates   The balance which exists at low temperatures is disturbed, when the temperature is raised, by increases in both the virulence of the infecting organism and the immunological responses of the host   In particular, antibody production is inhibited at temperatures below about 12° C

Summary

1. Active immunisation of frogs at 20° C. confers considerable protection against a lethal dose of virulent bacteria. No protection is afforded by immunisation at 8° C.

2. Natural immunity to infection is slightly greater in frogs and fish which have previously been kept at the lower temperature.

3. These conclusions assist in the reconciliation of previous anomalous findings on the affect of temperature in diseases of cold-blooded animals.

REFERENCES

BISSET, K. A.   .   .   .   .   .   .   1946.   This *Journal*, lviii, 251.
    ,, ...   1947a. This *Journal*, lix, 301.
    ,, ...   1947b. J. *Hyg.*, Camb., xlv, 128.

616 . 62—006 . 46—089 . 843

SURGICAL TRANSPLANTATION OF A CARCINOMA OF THE URINARY BLADDER

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(PLATES CIII AND CIV)

The possibility of surgical transplantation of malignant tumours is well recognised. It is, however, only rarely possible to observe the phenomenon carefully, and it is for this reason that it is considered justifiable to place the following case on record.

Case history

A male patient, E. S., aged 49, was admitted to the Westminster Hospital on 24.12.46, with a history of profuse hæmaturia for one month and an isolated attack of painless hæmaturia seven years previously which lasted for 48 hours. A diagnosis of carcinoma of the bladder was made after cystoscopy, and on 31.12.46 Sir Stanford Cade opened the bladder through a mid-line extra-peritoneal abdominal incision. A large amount of friable papillomatous tumour tissue was removed, and, after the base of the growth had been fulgurated, the wound was closed around a large catheter. The abdominal sinus remained patent until 17.2.47, when Mr Stanley Lee performed a second operation for transplantation of the ureters into the sigmoid colon as a preliminary to cystectomy. At the time of this operation a small nodule was noticed on the posterior surface of the anterior abdominal wall in the region where stitches had been inserted during the previous operation. This was removed for biopsy. After the operation the patient had a temporary period of relief, until he began a gradual downhill course and finally died on 18.5.47 of an ascending renal infection. On 22.4.47 a portion of the skin of the abdominal operation scar had been excised, as it apparently contained a nodule of growth.

Histology

The primary bladder tumour proves to be a typical transitional-cell carcinoma of papillary type (fig. 1). The biopsy from the anterior abdominal wall also shows tumour tissue (figs. 3 and 4), embedded in which is seen a small group

PLATE CIII

TRANSPLANTATION OF CANCER OF BLADDER
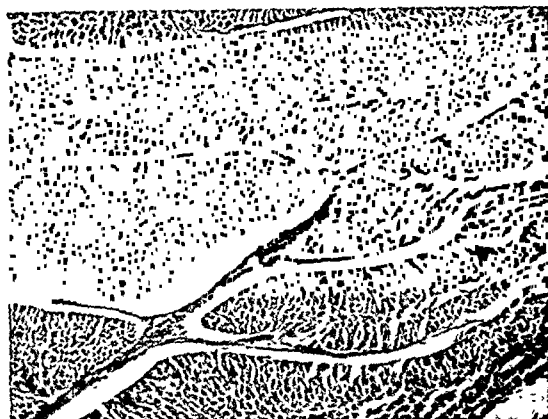
FIG 1 —Low power view of part of the bladder carcinoma removed at the first operation. ×60.
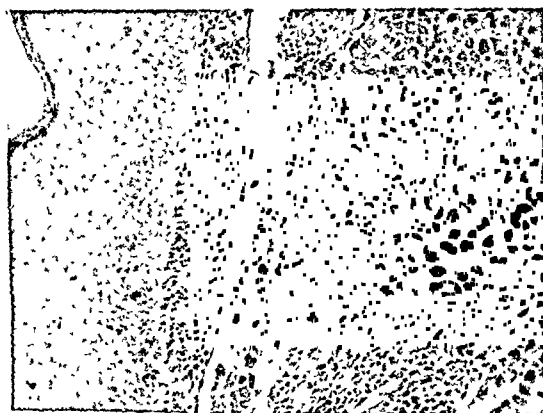
FIG 2 —High power view of the deposit (transplant) in the skin of the abdominal wound (second biopsy). ×140.

TRANSPLANTATION OF CANCER OF BLADDER
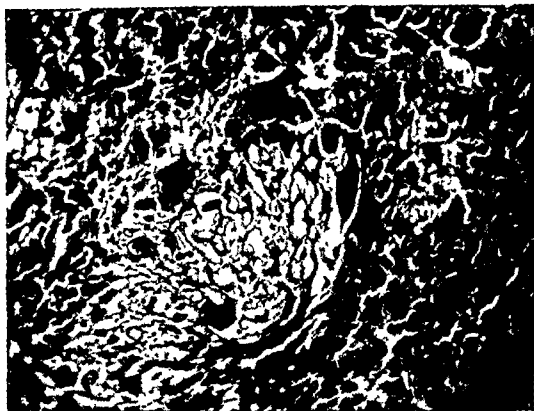
FIG 3 —High power view of stitch transplant into the posterior surface of the anterior abdominal wall (first biopsy), showing cat gut relics embedded in tumour tissue ×150
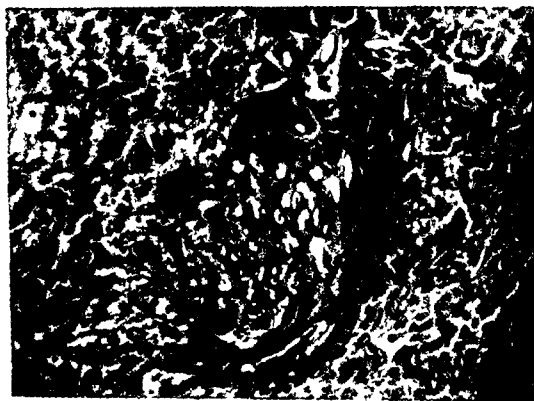
FIG 4 —The same field photographed through partially crossed Nicol prisms, showing the cat gut relics brightly illuminated ×150

of highly refractile, optically active bodies—obviously suture relics—surrounded by a foreign-body giant-cell reaction. The presence of these suture relics gives added support to the view that the malignant cells have been transferred to their new site by the stitch.

The biopsy of the abdominal scar skin (fig. 2) shows further infiltration of the dermis by anaplastic carcinoma cells, which extend right up to the deeper layers of the epidermis.

Discussion

Numerous references to surgical transplantation of tumours are to be found in the literature, and even those who have levelled criticisms against the theory of spontaneous implantation metastasis on epithelial surfaces have never denied the occurrence of accidental surgical transference. Willis (1934) presents a good review of the literature. Most of the cases reported are of transplants into an anterior abdominal wall scar following an intra-abdominal operation. Maybury and Dyke (1925-26) reported multiple growths in the scar resulting from an operation on what was apparently a simple papilloma of the bladder. The secondary deposits were considered to be malignant. Barnard and Robb-Smith (1945) refer to a case of Sir Bernard Spilsbury's where an adenocarcinoma of the liver was transplanted into the overlying skin following exploration with syringe and needle in order to exclude a liver abscess.

In the case here presented it is considered that both the deposits which appeared in the anterior abdominal wall following the original operation are examples of surgical transplantation. Evidence for this view is particularly strong in respect of the first biopsy, which shows a foreign-body reaction around suture relics in the midst of the carcinomatous deposit, and it would seem reasonable to conclude that the tumour removed at the second biopsy had also reached its site in the abdominal scar as a result of surgical transplantation.

Summary

A case of carcinoma of the bladder is presented in which transplantation of the tumour occurred both to the posterior surface of the anterior abdominal wall and, later, to the skin of the abdominal scar.

My thanks are due to Sir Stanford Cade and Mr E. Stanley Lee, the surgeons in charge of this case; and to Dr Peter Hansell of the Westminster Hospital Photographic Department.

REFERENCES

BARNARD, W. G., AND ROBB- 1945. Kettle's Pathology of tumours,
    SMITH, A. H. T.   3rd ed., *London*, p. 24.
MAYBURY, B. C., AND DYKE, S. C. 1925-26. *Brit. J. Surg.*, xiii, 377.
WILLIS, R. A. . . . . . . 1934. The spread of tumours in the human
                                     body, *London*, p. 95.

PLATE CV

FIG. 1.—Blood film from case 1 (splenectomised familial hæmolytic anæmia) showing basophilic granules in red cells. Leishman. × 690.

FIG. 2.—Same area as fig. 1, now showing siderotic in place of basophilic granules, after de-staining with eosin and then re-staining by means of the Prussian blue reaction. × 690.

FIG. 3.—Blood film from case 2 (splenectomised acquired hæmolytic anæmia) showing basophilic granules in red cells. Methylene blue. × 690.

FIG. 4.—Blood film from flexed-tail anæmic mouse showing basophilic granules in red cells. Methylene blue. × 645.

FIG. 5.—Same area as fig. 4, showing de-staining of granules after eosin treatment. × 645.

FIG. 6.—Same area as figs. 4 and 5, now showing siderotic in place of basophilic granules after re-staining by means of the Prussian blue reaction. × 645.

SIDEROCYTES

Fig 1

Fig 2
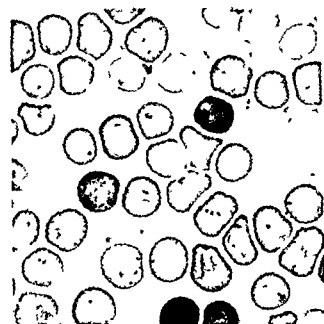
Fig 3
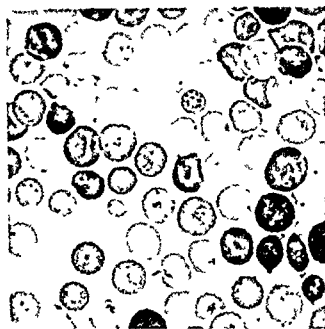
Fig 4
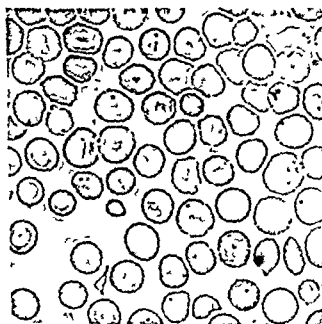
Fig 5

Fig 6

SIDEROCYTES
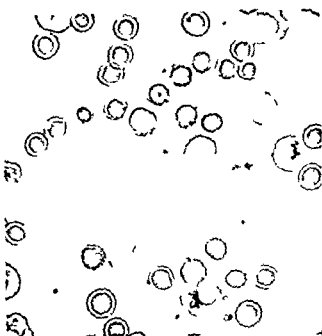
Fig 7—Blood film from case 5 (splenectomised acquired hæmolytic anæmia) showing siderocytes and siderotic granules in cytoplasm of some nucleated red cells the distribution is perinuclear in one cell Prussian blue   × 690
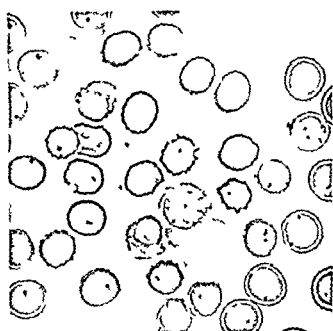
Fig 8—Sternal marrow film from case 1 showing siderocytes and siderotic granules in some nucleated red cells   The granules are absent from the more primitive cells Prussian blue   × 690

Fig 9—Blood film from case 4 (myelosclerosis and splenomegaly) showing scattered basophilic granules in many red cells and classical diffuse punctate basophilia in two prominent cells situated below and to the left of the centre Methylene blue   × 690
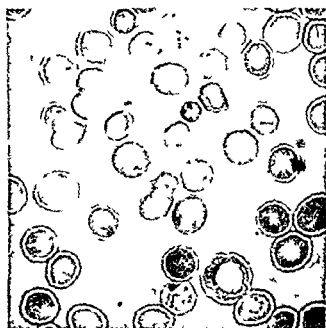
Fig 10—Same area as fig 9 showing siderocytes and a negative iron reaction of the punctate basophils   The nucleated red cell in the right lower corner shows a perinuclear distribution of siderotic granules Prussian blue   × 690

chromatic and polychromatic cells   Numerous siderocytes were demonstrated in another film submitted to the Prussian blue test

Fig 4 shows a methylene blue stained blood film, kindly given to us by Dr Gruneberg, of a four day old flexed tailed anæmic mouse   There are numerous basophilic granules of various sizes in the red cells   Fig 5 shows complete de staining of the granules by eosin   In fig 6 the same area re stained by potassium ferrocyanide and hydrochloric acid shows siderotic granules which can be identified with the basophilic granules seen in fig 4   As in case 1, not all the basophilic granules appear to contain stainable iron

Fig 7 (case 3) shows a blood film submitted to the Prussian blue reaction from a patient with acquired hæmolytic anæmia who had relapsed after splenectomy   In addition to scattered siderocytes, siderotic granules are present in the cytoplasm of some of the nucleated red cells, which were plentiful in the peripheral blood   In a proportion of the nucleated red cells the iron containing granules showed a perinuclear distribution , one example is seen in fig 7 Romanowsky preparations of this case showed basophilic granules in erythrocytes   They could not be readily identified in the basophilic cytoplasm of nucleated red cells   Fig 8, a film of sternal marrow from case 1 stained by means of the Prussian blue reaction, again demonstrates that siderotic granules may be found within the cytoplasm of nucleated red cells   They are absent from the more primitive non hæmoglobinised red cell precursors

Fig 9 (case 4) is a methylene blue stained blood film from a patient with myelosclerosis and splenomegaly (no splenectomy), whose peripheral blood contained nucleated red cells and showed classical punctate basophilia as well as siderotic basophilic red cell inclusions   Two prominent red cells in fig 9 show the numerous diffusely scattered small dots of punctate basophilia   These contrast with the few coarse and fine bodies seen in other red cells   Fig 10 shows the same area after eosin treatment and re staining by the Prussian blue method   The cells which showed diffuse punctate basophilia give a negative reaction for iron   The larger basophilic granules in the small cell near the centre of the figure give a positive iron reaction, as do the scattered granules and fine dots previously almost masked by cytoplasmic basophilia in adjacent red cells

The above findings confirm our impression that the red cells containing basophilic iron positive granules described by Pappenheimer *et al* were in fact siderocytes, and we have presented further evidence in figs 1, 3, 4 and 9 that siderocytes may show basophilic staining with methylene blue and the Romanowsky dyes   Whether all siderotic granules as revealed by Gruneberg's technique are basophilic remains to be seen   In addition to the negative reaction for iron of the diffuse punctate basophilia seen in fig 10 we found a negative reaction in Howell Jolly bodies   We cannot explain why the films showed fewer siderotic than basophilic granules (figs 2 and 6)   The iron content of the negatively reacting granules may be too small for detection by the Prussian blue reaction , possibly but we think less likely, they represent altered remnants of primitive cytoplasm as described for classical punctate basophilia   The demonstration (figs 7, 8 and 10) of the appearance of siderotic granules in nucleated red cells, apparently *pari passu* with the cells' acquisition of hæmoglobin, favours Gruneberg's hypothesis of their origin

Summary

A small series of photomicrographs of blood films is presented which demonstrates that siderotic granules in red cells may be basophilic

REFERENCES

CASE, R. A. M. . . . .   1943. *Nature*, clii, 599.
        „   . . . .   1946. *Proc. Roy. Soc.*, B., cxxxiii, 235.
CASE, R. A. M., LADAN, VERA N.,   1945. *Nature*, clv, 270.
    AND NUTT, MARJORIE
DONIACH, I., GRÜNEBERG, H., AND   1943. *This Journal*, lv, 23.
    PEARSON, J. E. G.
GRÜNEBERG, H.   . . . .   1941a. *Nature*, cxlviii, 114.
        „   . . . . .   1941b. *Ibid.*, cxlviii, 469.
        „   . . . . .   1942. *J. Genetics*, xliv, 246.
PAPPENHEIMER, A. M., THOMPSON,   1945. *Quart. J. Med.*, N.S., xiv, 75.
    W. P., PARKER, D. D., AND
    SMITH, KATHARINE E.

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ECTOPIC DECIDUA IN THE RENAL PELVIS

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(PLATE CVII)

The frequency with which a diagnosis of ectopic decidua is made depends to some extent on what is regarded as ectopic decidua. If the term is taken in its widest sense to include the occurrence of decidual elements at any site other than that of the implantation of the ovum, the condition is fairly common. However, it would be the formation of decidua in the uterus during an ectopic pregnancy that would account for many such diagnoses. An even larger number of cases could be collected if the decidual reaction of the endocervical stroma during pregnancy were included. This occurrence is not as widely known as it might be, probably because there is not often the opportunity to obtain material from the cervix for histological examination during pregnancy. It might, however, be worth while to record in passing that we have obtained in this department a large series of cervical biopsies, taken on the occasion of curettages for incomplete abortion, and have seen at least some evidence of decidual reaction in practically every case. It is also a regular finding in cervical polyps removed during pregnancy. The practical importance of these observations lies in the fact that isolated collections of decidual cells have not infrequently been mistaken for carcinoma.

It seems better, therefore, and more in keeping with general usage to restrict the range of the term " ectopic decidua " to cases occurring outside the uterus. The condition is then fairly rare, the ovaries being most frequently involved, but observations of its occurrence over a wide area of the parietal and visceral peritoneum and its underlying structures have been recorded.

It is probably no coincidence that the pattern of the distribution of ectopic decidua follows rather closely that of endometriosis. There is first the likelihood that a number of cases of ectopic decidual formation actually occur in areas of endometriosis. The decidual reaction of the stroma can be so extensive that it makes any glandular elements which may be present quite inconspicuous. Secondly, both conditions obviously require for their occurrence two factors, a suitable soil and a suitable stimulus. The " soil " is the same for both conditions —a mesenchymal tissue capable of responding with the formation of ordinary

ECTOPIC DECIDUA IN THE RENAL PELVIS

Fig 1 —Section through renal pelvis showing edge of decidual plaque (to left) ×50
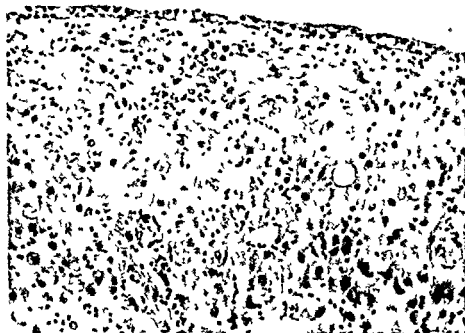
Fig 2 —Portion of decidual plaque with overlying thin layer of renal pelvic epithelium ×150
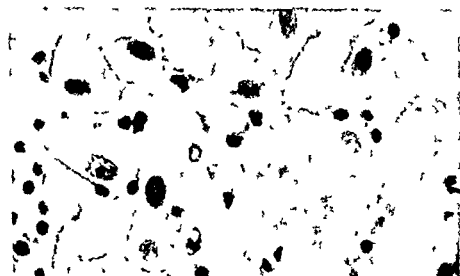
Fig 3 —High power view of part of fig 2 ×600

or decidual endometrial stroma. Such tissue is provided wherever derivatives of the cœlomic epithelium—which early in embryonic life gave rise to the mesonephric ridge—have retained some of their original properties This reasoning certainly implies that one follows R. Meyer's and E. Novak's ideas (Novak, 1947) that endometriosis originates at sites where this is found. Sampson's (1940) hypothesis, always difficult to accept, has become quite untenable since Smith and Smith (1946) have proved that menstruating endometrium contains severely toxic substances The stimuli for the development of endometriosis and ectopic decidua are, if not identical, at least very closely related. Gonadal hormones are involved in both conditions, but in the former the accent lies on œstrogenic compounds, whereas, for the latter a strong progesterone action seems essential.

In both conditions, theory is sometimes confronted with the difficulty of having to find an explanation for a most unusual site of the lesion. One has only to think of the famous case of endometriosis of the arm, but in this instance Gruenwald (1942) seems to have provided a plausible explanation.

With regard to ectopic decidual formation, the following observation is unique as far as I have been able to ascertain

A woman, aged 33 years, under the care of Dr J. W. Johnstone, was found to have severe hæmaturia during the fourth month of her third pregnancy. Further investigation disclosed the presence of a right sided hydronephrosis, and as the bleeding from this kidney continued unabated, nephrectomy was performed by Dr H. Moore

The kidney was only slightly enlarged and of normal configuration. The renal pelvis was grossly dilated and only a small rim of kidney tissue surrounded it Scattered throughout the renal pelvis were a number of whitish plaques of about one centimetre in diameter. They were only a few millimetres in height and had a smooth surface. Microscopically they were lined by a greatly thinned epithelium, the thick transitional epithelium seen elsewhere in the pelvis being reduced to one or two layers of cells (figs 1 and 2). The bulk of a plaque was composed of cells which had all the characteristics of decidual cells (figs 2 and 3)

This finding was so surprising, and, as a check of the literature showed, so unusual, that every attempt at arriving at another interpretation was made However, no other conclusion seemed possible and it is necessary to see how this occurrence can be reconciled with the theoretical requirements discussed earlier Can the renal pelvis be regarded as a "suitable soil"? Its anlage develops as a bud from the caudal end of the mesonephric duct; the Müllerian or paramesonephric duct develops either from the cranial portion of this anlage or at least from the same blastema from which the mesonephric duct originated earlier Part of this blastema is the overlying cœlomic epithelium, and it is known that its progeny in the form of the peritoneal mesothelium can undergo decidual transformation. A connection can therefore be established, though a remote one, but probably it is just this remoteness that accounts for the extraordinarily rare occurrence of the condition here recorded.

REFERENCES

GRUENWALD, P.   .   .   .   .   1942. *Amer. J. Obst. and Gyn*, xliv, 470.
NOVAK, E .   .   .   1947. Gynecological and obstetrical pathology, 2nd ed , *Philadelphia* and *London*, p. 473.
SAMPSON, J. A. .   .   .   .   1940. *Amer. J. Obst and Gyn*, xl, 549.
SMITH, O. W., AND SMITH, G. VAN 1946. *J. Clin. Endocrinol*, vi, 483.
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THE MECHANISM OF SWARMING OF PROTEUS

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(Plates CVIII and CIX)

In our work on the inhibition of swarming of Proteus by detergents (Lominski and Lendrum, 1942) we were struck by the complexity of the swarming process itself and the present study is an investigation of its cause and course.

Swarming as seen under a low power may be briefly described as follows. On solid media Proteus grows at first without swarming, which appears some 3-8 hours after inoculation, the time of onset depending mainly on the load of inoculum (Moltke, 1929), the concentration of the agar and the lag-time of the culture. Light inocula from very young actively motile cultures occasionally result in a swarming growth without the appearance of a solid primary stroke; the ring phenomenon occurs secondarily as usual. The onset of swarming is sudden; clumps of organisms shoot out from the periphery of the colony and migrate a considerable distance—occasionally as much as 1 cm.—turn slightly back and settle. Practically all migrating clumps settle more or less at the same distance from the colony and thus form a ring where stationary growth begins again. The swarming phase lasts for some 10-30 minutes and is followed by a period of stationary growth of some 3-6 hours; the whole process is then repeated up to the limit of the available surface. Meanwhile the space between the zones of stationary growth becomes gradually filled in but never quite to the thickness of the zones themselves; this gives to a Petri-dish culture the appearance (fig. 1) of alternating positive and negative rings as described by Epstein (1919) and studied by Russ-Münzer (1934-35). In the early stages it is actually possible to demonstrate that many points in a negative zone are sterile. To sum up, swarming does not appear to be continuous in occurrence or haphazard in direction; it is periodical and directed away from the place of stationary growth.

The cause of swarming has apparently excited little interest in the past; the fact that swarming of Proteus is a hindrance in isolating other organisms has, on the other hand, focussed the attention of innumerable workers on its suppression. Among those interested in the phenomenon itself, Cantu (1911) was apparently satisfied that the very active motility of Proteus is sufficient explanation of swarming; Seiffert (1920), who thought that swarming is suppressed by malnutrition, regarded it as a normal feature of healthy individuals belonging to the genus Proteus. Moltke, on the other hand, called it a deliberate (zielsicher) action following on " hunger " and " aiming at better life conditions ". The possibility that swarming might be due to negative chemotaxis was raised for the first time by Russ-Münzer, but having failed to extract an active substance from the bacterial bodies she concluded that swarming is due to starvation and is part of the life cycle of Proteus on solid media.

Neither the theory of active motility nor that of starvation, however, seems to us capable of explaining the orientation of swarming or its periodicity. A more likely explanation would be that swarming is due to negative chemotaxis exerted by the metabolites of the organism, these metabolites being elaborated and accumulating at the site of stationary growth. When a certain critical concentration is reached they stimulate swarming of some of the organisms,
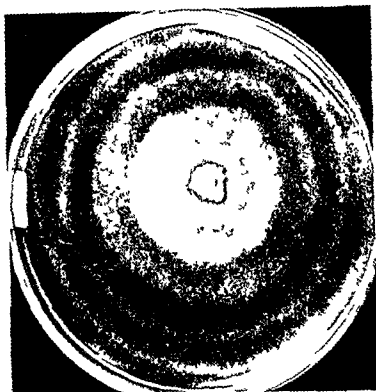
Fig 1—Petri dish culture 18 hours after inoculation of the agar surface at the centre of the circle, showing central mass of growth and concentric rings of growth in the swarm which extends to the limit of the available surface   × ¾
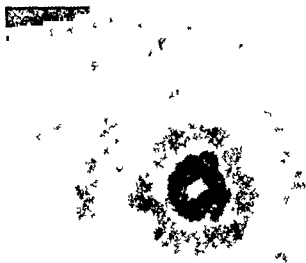
Fig 2—Petri dish culture 24 hours after an inoculation of the agar surface in the form of a rectangle, this shows the central enclosed agar surface free of swarm and the surrounding surface covered by typical swarm with rings   × 1 1

possibly the younger or the more motile. The metabolites diffuse into the medium and form a gradient of decreasing concentration. Swarming takes place down this gradient. In the zone immediately surrounding the stationary growth the concentration of metabolites is such that the organisms proceed across this zone without stopping; this territory is, as it were, "taboo" for settling. When, in their progress down the gradient, they reach an area relatively free of metabolites, settling and stationary growth are again possible and a ring of growth is formed—the positive ring. On such a hypothesis the orientation of swarming, its periodicity and the consequent formation of rings could be explained.

Experimental observations

All the experiments described below have been carried out with 24 strains of Proteus (12 *P. vulgaris* and 12 *P. mirabilis*), all fairly recently isolated.

Expt. I. The following experiment was devised to test the idea that swarming occurs down the gradient of metabolites. On the surface of an agar plate a heavy inoculum was made from a solid culture, in the form of a ring or quadrangle 3·4 mm. wide, care being taken to leave the inside free of inoculum. It appears justifiable to assume uniform diffusion of the metabolites and, since agar is a fairly solid colloid, it is probable that a higher concentration of metabolites will be built up inside the ring than outside it. If this idea is correct, swarming should occur more readily outwards than inwards, or possibly not occur inwards at all. The experiment (fig. 2) showed that, provided the circle or quadrangle is not too large, the inoculum sufficiently heavy and the agar not too concentrated for diffusion (2 per cent.), the inside of this "magic circle" can remain sterile for 48 hours or at most show only a thin veil of swarming organisms, whereas the rest of the agar surface is covered by a thick overall swarm. This seems to prove the hypothesis of negative chemotaxis and the directional effect of the gradient of metabolites on the swarming.

Expt. II. In an attempt to explain the formation of the negative rings the following experiment was carried out. A stroke was inoculated towards one edge of a Petri dish from a culture known to swarm fairly regularly after 3 hours at 37° C. under our experimental conditions. Before the expected onset of swarming—say after 2½ hours—the primary stroke was isolated from the rest of the agar by a cut parallel with and close to it (fig. 3). The free area was then divided into two segments by a cut at right angles to the previous cut. One of the isolated segments was left as control to show that swarming from the primary stroke had not occurred during the first incubation period. On the other segment several secondary strokes of inoculum were made parallel to the primary stroke and at increasing distances from it; the dish was then re-incubated. After 24 hours in a successful experiment the control segment is sterile. The primary stroke shows stationary growth and swarming over the whole portion of its agar. On the segment with the secondary strokes there is generalised swarming; the strokes more distant from the primary show distinct stationary growth, but at the site of the nearest secondary stroke there is not a trace of stationary growth (fig. 3). In this experiment the organisms (at least those of the first secondary stroke) were inoculated on to what we assumed was a metabolite gradient, which in the previous experiment was shown to influence the direction of swarming. The results indicate that on such a gradient there is swarming, but stationary growth does not occur. It can also be concluded that the metabolites diffuse rapidly into the agar and before the onset of swarming.

Expt. III. In an attempt to obtain the active metabolites cell-free, a 24-hour broth culture of Proteus was filtered through a Chamberland L3 candle. Two separate strips of agar each about 8×2 cm. were prepared and placed in a Petri dish and a short trench holding about 0·5 c.c. was made at the end of

each. The filtrate was pipetted into one and, by way of control, broth or *Bacterium coli* filtrate into the other. After allowing some 30 minutes for diffusion, both strips were inoculated about 3 mm. from the edge of the trench with Proteus suspended in saline—this in order to minimise the transfer of pre-formed metabolites. The strips were left at room temperature. With an active filtrate swarming starts at once and can be detected by observation under a low power or by making subcultures from distant areas, whereas swarming in the control strip takes several hours to appear (fig. 4). Also, on the Proteus filtrate strip the swarm is all away from the trench while on the control strip the swarm covers the whole available surface. It should be said that the filtrate experiments often failed, sometimes for reasons we were able to explain : thus Seitz filters are unsuitable, filtrates which have been kept too long are inactive, and a too concentrated agar prevents diffusion ; but many failures could not be accounted for. However, since early swarming never occurred in the controls, the positive results of 46 out of 98 experiments are considered significant. Thus a filterable substance can be obtained capable of initiating swarming.

Expt. IV.—Finally the following experiments were carried out in order to investigate the possible effect of malnutrition on swarming. An agar plate was allowed to become overgrown for 24 hours with a non-maltose-fermenting Proteus ; the surface was thereafter scraped with a glass slide and the centre inoculated with a maltose-fermenting variety, the biochemical difference allowing the recognition of swarming of the second inoculum. Under these conditions growth is very poor, and swarming, as tested by subculture, completely absent. However, if such plates before the second inoculation are left for a few days on the bench, or are exposed to ether vapour for 6 hours, or are re-melted, they lose their anti-swarming properties without improvement in nutrient properties. Further, media overgrown with *Bact. coli* or staphylococci inhibit growth but do not inhibit swarming ; growth on such media is delicate, but swarming, though almost invisible to the naked eye, can be detected by culture. It is thus clear that inhibition of swarming and inhibition of growth are not due to the same factor and that malnutrition alone does not inhibit swarming. The absence of swarming on media recently overgrown with Proteus could, in the light of previous experiments, be attributed to lack of a gradient, such media being uniformly permeated with the active metabolite. On such media the restricted growth does not supply enough of the active metabolite to create a substantial excess and thus superimpose a local gradient. We have already shown that swarming occurs down a gradient and that stationary growth does not incur on a gradient ; it can now be added that stationary growth can occur even in the presence of a high concentration of metabolites if the concentration is spatially uniform—on a plateau as it were—and that, in the absence of a gradient, there is no swarming. These experiments also show the labile nature of the active metabolite or metabolites.

Little information has so far been obtained about the nature of the active metabolite ; on the possibility that it might be merely alkalinity, sodium hydroxide ($N/50$) and ammonia (5 per cent.) were tested but had no effect on swarming. The ready disappearance of the active metabolite suggests that it may perhaps be a volatile substance.

Certain well-known phenomena may be reviewed in the light of these results. Thus it becomes understandable why the higher the water content of the agar the greater the width of the negative rings formed by Proteus, because diffusion will obviously be more rapid in the more hydrated agar and the metabolite preventing stationary growth will affect a wider zone. Again, Russ-Munzer noticed that on solid media Proteus undergoes a cyclic change of morphology coinciding with the alternating phases of stationary growth and swarming (the organisms are short in stationary growth and filamentous in swarming),

SWARMING OF PROTEUS
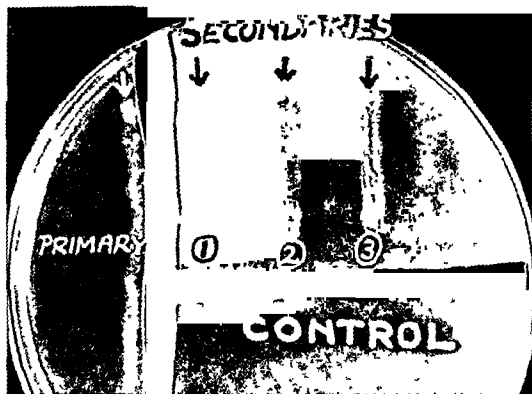
Fig 3 —Petri-dish culture showing by arrows the site of the secondary strokes made
on the segment isolated by ditching before the swarm from the primary stroke
had reached it No stationary growth is visible at the site of the secondary stroke
nearest to the primary. (For fuller details see text).   1 1
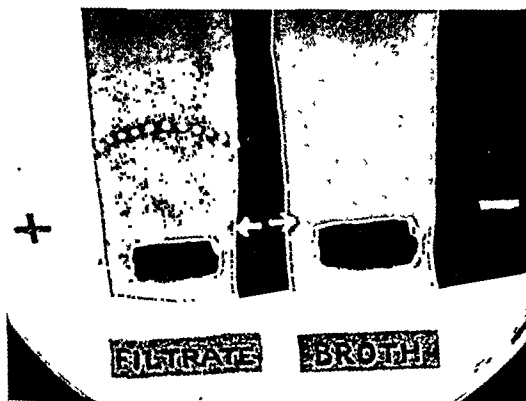
Fig 4 —Agar strip with trough containing Proteus filtrate, showing by arrows the site
of stroke inoculation At room temperature, within 20 minutes of inoculation,
Proteus had reached the dotted line (demonstrated by subculture)   ×1 5

but that in fluid cultures no such periodic variation occurs and short rods only are present   (In cultures older than 48 hours we noticed the gradual appearance of long forms)   Filamentous forms of bacteria are frequently the result of a damaging effect of metabolites on cell division , the local accumulation of metabolites in solid media as opposed to their uniform diffusion in fluids is a tempting explanation for the phenomenon observed by Russ Munzer   Finally, it is well known that growth in soft or semi solid media may stimulate the motility of certain flagellate organisms which show no motility in fluid cultures, and this effect may well be due to the formation in semi solid media of a gradient of metabolites, presumably exerting a negative chemotaxis similar to that of Proteus   Such a gradient cannot exist in fluid cultures

Summary

Swarming of Proteus is motility stimulated and orientated by the negative chemotactic action of its metabolites   Swarming occurs only under conditions affording a spatial gradient of these metabolites   The active substance can be obtained cell free

REFERENCES

CANTU, C	1911	*Ann Inst Pasteur*, xxv, 852
EPSTEIN, E	1919	*Zbl Bakt* , Orig , Abt I , lxxxiii, 255
LOMINSKI, I , AND LENDRUM, A C	1942	This *Journal*, liv, 421
MOLTKE, O	1929	*Zbl Bakt* , Orig , Abt I , cxi, 399
RUSS MUNZER, A	1934 35	*Ibid* , Orig , Abt. I , cxxxiii, 214.
SEIFFERT, W	1920	*Z Immunitatsforsch* , I Teil Orig , xx, 551

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SOLITARY ADENOMYOMA OF THE UTERUS

MAGNUS HAINES

Chelsea Hospital for Women, London

(PLATE CX)

The tumour of the uterus here described is interesting not only in that it caused symptoms during the puerperium but also because of its probable nature—solitary adenomyoma

CASE REPORT

Clinical summary

Mrs S P , age 33, the wife of a machinist, first attended hospital in August 1943 because of a miscarriage   Before and after this she had no painful periods   In August 1946 she was delivered of a still born infant weighing 8 lb 2 oz , and remained in bed for six weeks   Towards the end of September and in early October she was again in bed because of hæmorrhage—possibly the return of menstruation   At the end of October she attended hospital complaining of pain

in the abdomen and occasionally in the vagina. The uterus was enlarged to the size of a 14 weeks' pregnancy. The Friedman test was negative. She was examined again in three weeks, when the uterus was found to be larger. She stated that her period had begun 13 days before and had lasted 6 days—a normal period. She still complained of pain. Hysterectomy was performed. The ovaries appeared healthy.

She has one child, age 10, alive and well, and there had been three miscarriages.

Morbid anatomy

The specimen is a complete uterus without appendages. It is asymetrically enlarged and measures 13 cm. in length. There is a globular tumour in its wall, situated posteriorly and to the right. The tumour measures 8 cm. antero-posteriorly and is 6 cm. in diameter as seen in fig. 1. In the recent state it was soft to the touch but resilient as sorbo rubber. On bisecting the uterus in the frontal plane a honeycomb or sponge-like tumour is seen. It appears to have arisen in the uterine isthmus and to have expanded laterally and posteriorly. It appears to be encapsulated, but strands of muscular tissue are also seen within the tumour between the cavities. The tumour is intramural and has caused elongation and distortion of the uterine cavity. The endometrium is uniform in thickness (3 mm.). It is not possible to "shell out" the tumour from the myometrium. No myomata are seen in the specimen. Blocks for section were taken from the endometrium above and over the tumour, and from the tumour itself.

Microscopical examination

Endometrium. The stroma and glands show the proportion and pattern normal for the proliferative (oestrogenic) phase. No direct continuity is traced between the normally situated corporeal endometrium and that of the tumour.

Capsule. The apparent "capsule" consists merely of compressed myometrium.

Tumour. The fenestrations in the tumour, of various sizes up to 2 mm. diameter, are all lined by cubical or low columnar epithelium resembling corporeal epithelium. These glandular structures are embedded in a spindle cell stroma, the elements of which greatly outnumber the epithelial by comparison with normal endometrium. The stroma is for the most part exceedingly dense and hyperplastic, like that seen in the normal stratum basalis of the endometrium. In other areas the stroma cells are smaller, less deeply staining and more sparsely arranged, resembling the cytogenous layer usually encountered in endometriosis. Over all, the pattern consists of innumerable epithelial lined spaces, each surrounded by its own, often thick, investment of stroma.

That this tumour is formed by endometrial permeation of the myometrium is apparent from the discovery of several strands of smooth muscle fibres lying amongst the stroma (fig. 2). Here the appearance conforms with that of endometriosis and, for those retaining the old idea of adenomyoma, there are present the two criteria, endometrium and myometrium.

The content of the cystic spaces varies. Much has been lost in fixation. Many spaces contain "mucus", and in some instances mononuclear cells are seen in the lumen. These have the general appearance of swollen phagocytes. In no case did they contain iron-reacting pigment. No "pseudo-xanthoma" cells were seen in the stroma.

COMMENTARY

Although myoma of the uterus is a common tumour and its study, both pathological and clinical, has been in progress for many years, little attention has been paid, until comparatively recently, to those which contain cavities

ADENOMYOMA OF UTERUS
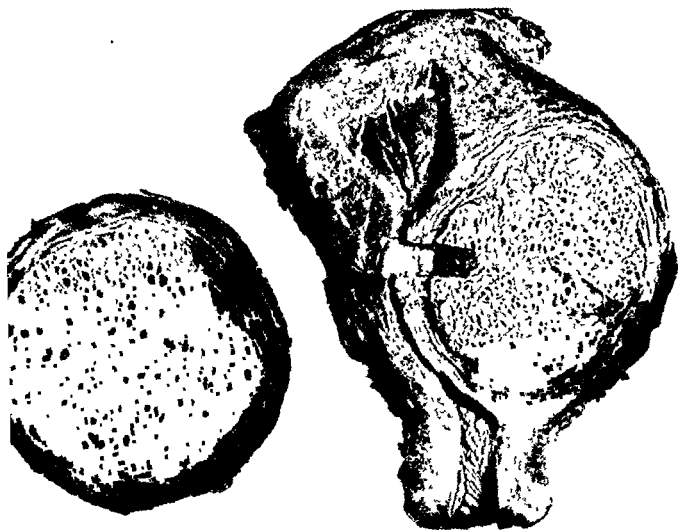
Fig. 1—Right, the uterus bisected in coronal section, showing the tumour and the distortion of the uterine cavity. Left, a slice of the tumour from the back of the specimen. ×⅕.

Fig. 2—
... stroma ...

... endometrium intra- ...

lined by an epithelium It should be pointed out that unqualified state ments (Curtis, 1933) that myomata occur in which epithelial lined cystic cavities are present are liable to misinterpretation Many myomata in which telangiectatic or lymphangiectatic cavities occurred have been incorrectly described or copied, as containing cysts lined by " epithelium " instead of, more accurately, endothelium However, in 1882 we find Babes describing an intramural myoma of the fundus uteri containing cysts lined by cubical epithelium Other accounts followed, but it was the outstanding work of von Recklinghausen (1896) and of Cullen (1908) which created world wide interest in this subject They introduced the concept of adenomyoma and were concerned with theories of ætiology which it is unnecessary to consider here It was their contention that such tumours be described as comprising both endo metrium (glands and stroma) and smooth muscle (myometrium) Gradually it was realised that the neoplastic view of the muscular component was un tenable and in 1922 we find Blair Bell introducing the term endometrioma Finally, this was changed in favour of endometriosis by Sampson (1927), who was opposed to the whole idea of neoplasia In the course of these studies many instances of endometriosis were found to occur outside the uterus (adnexa etc), so that there arose the division into internal and external endometriosis Many workers nowadays use the terms adenomyoma and internal endometriosis as synonyms

In the case now reported, a clinical diagnosis of myoma was made, and the contour of the uterus, when removed, was in favour of this The consistency of the tumour aroused suspicion , although it was realised that degenerating myomata are soft, they are not usually of a spongy nature as in this case Inspection of the cut surface aroused further suspicions This showed hundreds of tiny cystic spaces with clear smooth linings The cyst walls did not collapse after section A cystic myoma, on the other hand, shows more or less collapse of the cyst walls and the lining is often rough or even shaggy Unlike a myoma, the tumour would not ' shell out ", and strands of fibromuscular tissue are seen coursing into the tumour Examination of the section shows clearly that the capsule, if such it be called, is merely compressed myometrium

There are no myomata elsewhere in the specimen nor is there any evidence to support the idea that the tumour is a genuine adenomyoma, i e in the sense defined by Stewart (1935), who now maintains (personal communication, 1947) that endometrial invasion, if localised, can stimulate a discrete muscular hypertrophy comparable to myomatous overgrowth

It may be remarked that a characteristic of this tumour is the considerable preponderance of stroma over epithelium As a rule, in endometriosis the proportions are reversed and stroma in any quantity is often hard to find But in this case there are special circumstances, namely, the recent full term pregnancy During this phase there would be considerable decidual reaction in the stroma of the tumour and it is probable that hyperplasia of stroma has persisted Each epithelial acinus has its own, often very thick, " cylinder " of stroma From the natural history of endometriosis, cyst development is to be expected This case appears to be unique in its generalised microcystic form Uterine adenomyomata exhibiting quite large cystic spaces have been described from Cullen's time onwards

SUMMARY

The clinical and pathological findings are described in a case of discrete adenomyoma of the uterus, a type of tumour rarely encountered during the puerperium In this instance hysterectomy was performed three months after the delivery of a still born infant

Illustrations prepared by the department of medical photography, Westminster Hospital

REFERENCES

BABES, V. . . . . 1882. *Allg. Wiener med. Zeitung.,* xxvii, 36.

BELL, W. BLAIR . . . . . 1922. *J. Obst. and Gyn. Brit. Empire,* xxix, 443.

CULLEN, T. S. . . . . . . 1908. Adenomyoma of the uterus, *Philadelphia* and *London.*

CURTIS, A. H. . . . . . . 1933. Obstetrics and gynecology, *Philadelphia* and *London,* vol. ii, p. 790.

RECKLINGHAUSEN, F. VON . . 1896. Die Adenomyome und Cystadenome der Uterus- und Tubenwandung, *Berlin.*

SAMPSON, J. A. . . . . . . 1927. *Amer. J. Obst. and Gyn.,* xiv, 422.

STEWART, M. J. . . . . . 1935. *In* TEACHER's Manual of obstetrical and gynæcological pathology, *London,* p. 236.

OBITUARY NOTICES OF DECEASED MEMBERS

Louis Cobbett

(Born 15th May 1863. Died 9th March 1947)

(PLATE CXI)

LOUIS COBBETT was the third son of Arthur Cobbett of Weybridge, and was educated at Lancing College (1875-81), where he became a school prefect. In 1881 he proceeded to Trinity College, Cambridge, and took the Natural Sciences Tripos in 1884. Though short in stature and light in weight, Cobbett was an excellent oarsman. He oared " bow " in the First Trinity first boat in the May races in 1883. At that time racing took place on six evenings in one week. This boat started second, " went down " on the first night and " rowed over " on the other five. In 1885 he rowed " No. 7 ". This boat started fifth and " rowed over " every night. Cobbett was very enthusiastic about swimming and skating but never took any interest in ball games.

In the academic year 1884-85, he passed the 2nd M.B. examination and attended courses in medicine and surgery, thus coming under the influence of Sir George Humphry (1826-1896) who, with Sir Michael Foster and Sir George Paget, was one of the founders of the modern School of Medicine in Cambridge. Humphry " was a magnetic teacher, employed the Socratic method and, keen observer himself, urged this attitude on his pupils ". Cobbett, who was a good raconteur, had many stories to tell of Humphry's ways and skill in repartee. For example, on one occasion when Humphry was an examiner in London, a candidate, seeing a rather shabbily dressed old man following a short distance behind a group of patients on their way to the Examination Hall and taking him for a patient, approached him with a half-crown in his hand and said in a whisper, " What is the matter with you, my man ? " Humphry gazed at the half-crown and shook his head. A second half-crown was added, but he still shook his head. When a half sovereign was substituted Humphry pocketed the coin, put a hand on each side of his mouth and whispered the name of some disease. Some candidates who were well acquainted with Humphry watched the episode from a distance.

About this time there was a controversy between those who were of opinion that the bowel should be evacuated daily and those who thought that longer intervals—up to a fortnight—might, at any rate in some persons, be regarded as normal and compatible with health. Humphry held the latter view, his house-surgeon the former, and they often discussed the matter in the presence of students. One

day a healthy-looking farm lad suffering from some injury appeared at out-patients. On Humphry asking him how often he went to the closet the boy replied " Once a week, Sir ". Humphry was delighted and looked at his house-surgeon with an expression which seemed to say, " Have you ever seen a more healthy looking lad than this ? " The house-surgeon, realising that the case would often be brought up against his view, asked " On which day ? " Receiving the reply " On Sundays, Sir ", the house-surgeon said " What about other days ? " The answer was " Anywhere behind the hedge, Sir ". Cobbett and others were watching from a platform on which students attending " out-patients " used to stand.

One of the best of Humphry's repartees has often been related in different ways, but Cobbett, who was present, gave the following account. Humphry, who was tall and thin, sat perched like a hawk on a high stool and his class, consisting of about eight students, sat in a semicircle before him. After asking a question he would point at one of them with his finger. On one occasion, after a difficult question, the finger, passing along the class in turn, skipped one member who seldom answered. The man looked annoyed but the finger passed on till it reached the last man and then suddenly switched back on to the one who had been omitted. To Humphry's evident astonishment, the correct answer was given. The man, still more annoyed, said " You seem to be surprised, Sir George ". Humphry replied, " So was Balaam when his ass spoke ".

In 1885 Cobbett went to St Thomas's Hospital, where later he became house-surgeon to the late Sir William MacCormac. He obtained the Conjoint Diploma in 1890, the F.R.C.S. in 1891 and the Cambridge M.B. in the same year. He took the M.D. degree in 1899 with a thesis " On the nature of the action of antitoxin ".

He returned to Cambridge in 1893, where he lived in lodgings at 2 Round Church Street, and, in the same year, was appointed demonstrator of pathology under C. S. Roy, the first professor of pathology in the University, at a stipend of £100 a year. In 1894 he resigned the demonstratorship on being appointed John Lucas Walker Student, a post which he held till 1897. Amongst the previous holders of the demonstratorship or studentship or both were Almroth Wright, Humphry Rolleston, W. Hunter, J. G. Adami, J. Lorrain Smith and A. A. Kanthack.

Cobbett's first paper was written in collaboration with W. S. Melsome and described extended experiments on local and general immunity produced in animals by inoculation with streptococci obtained from cases of erysipelas. He also began his studies on diphtheria, which included an experimental enquiry into the various methods of immunising horses for the production of antitoxin, the effects in patients of the newly introduced antitoxin treatment and the cultural characters of the diphtheria bacillus and organisms resembling it, and he published papers on these and other subjects.

PLATE CXI

After a visit to the Pasteur Institute in Paris, he was engaged during most of 1897 in making and testing diphtheria antitoxin in the laboratory of Messrs Burroughs Wellcome. In 1898 he returned to Cambridge and worked in the pathological laboratory till 1902 without holding any official post but assisting in the teaching of students. During this period he continued his studies on the bacteriology of diphtheria, and he introduced a solidified alkaline serum medium for the cultivation of material from throat swabs which had many advantages over the media commonly employed, and a very rapid and satisfactory way of staining the organisms in dilute Loeffler's methylene blue. He also collected evidence on the clinical effects of the antitoxin treatment of cases of diphtheria and began to study the ways in which the disease spread, especially among school children. In 1899 he noted that the toxicity of the serum of different horses for guinea-pigs varies greatly and that the susceptibility of different individual guinea-pigs varies even more. He noted the rapidly fatal results in some of the guinea-pigs and the great post-mortem distention of the lungs. He also observed that the toxicity was not destroyed by heating to 65° C. and was little affected by passage of the serum through a Martin gelatin-choked filter. In 1900-01, considerable outbreaks of diphtheria in Cambridge and Colchester gave him the opportunity of tracing the spread of the disease by the bacteriological examination of patients and of school, family and other contacts. He not only isolated the diphtheria bacilli in pure culture from almost every individual patient and contact found to be infected, but also investigated their virulence for guinea-pigs. Further, in order to check the spread of the disease by carriers, he arranged for their isolation in a building away from the isolation hospital until the bacilli seemed to have disappeared from their throats and noses as evidenced by three consecutive negative examinations of cultures. These were the first large-scale investigations of their kind.

Between 1892 and 1902 Cobbett worked in a small upstairs room, divided by match-boarding from other rooms, in the old pathological laboratory. This rather small two-storied building, which had been erected as a chemical laboratory in 1786, had housed at one period anatomy, botany, chemistry, mineralogy, mechanics and physic, and parts of it were in a dilapidated state, especially that occupied by the department of surgery, which was badly affected by dry-rot. In 1901 about half of the building was pulled down to provide part of the site for the new medical school, which was opened by King Edward VII in 1904. For the next 24 years the department of pathology occupied a considerable part of that building. It removed to the present building on the Downing site in 1928.

I first got to know Cobbett in the long vacation term of 1901 when, after qualifying, I returned to Cambridge wishing to learn a little more about pathology, which, with kindred subjects, appealed

to me more than practice, and joined a very small advanced class advertised to be given by Cobbett. A considerable outbreak of diphtheria was in progress in the neighbourhood, and the class devoted much of its time to observing Cobbett at work, going over cultures from swabs, purifying and identifying organisms from the cultures, observing the effects of inoculation of animals, making media, hearing the reports of the sanitary inspectors on various matters and of the doctors on the effects of antitoxin treatment,* following up patients and contacts and keeping records of all such matters. We were, in fact, gaining experience of all the necessary procedures, both technical and administrative. Cobbett's insistence on scrupulous care in regard to every detail and his enthusiasm and readiness to discuss every aspect of the outbreak, as well as the care he bestowed on the other subjects treated in his course, made a great impression.

Soon afterwards he was asked by the town council of Colchester for his help in dealing with an alarming outbreak of diphtheria, and Cobbett asked me to go there as his assistant and to carry on whenever he was absent. Next year, with the assistance of Professors Sims Woodhead and Nuttall, who had recently come to Cambridge, he obtained for me the appointment of bacteriologist to the borough of Cambridge and the surrounding districts. I am therefore almost entirely indebted to him for a start in a scientific career.

In 1902 Cobbett was appointed a scientific investigator to the Royal Commission on Tuberculosis with charge of one of the experimental farms. He therefore went to live in Stansted, and his sister Effie kept house for him from that time till her death a few years ago. The well-known account of his work for the Commission was published in 1907 in an appendix of 1200 pages to the Royal Commission's Report. It was illustrated with numerous photographs and figures by himself.

For one academic year (1906-07) he held the professorship of pathology in the University of Sheffield, but, preferring to live in Cambridge, he resigned and returned to Cambridge, where he was appointed university lecturer in pathology † in 1908, a post he held till 1929. During this period he taught bacteriology with great enthusiasm and published several papers and one book, chiefly on tuberculosis, with special reference to its method of spread. In October 1921 he was awarded the Weber-Parkes medal for the prevention and cure of tuberculosis by the Royal College of Physicians. After retiring from the lectureship, he continued to work in the pathology department at Cambridge for several years and lectured on tuberculosis to students taking part II of the Natural Sciences Tripos up to 1943.

In his younger days Cobbett was an excellent figure skater and for

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* At that time some of the practitioners were opposed to antitoxin treatment, calling it "bending the knee to the medicinal Baal".

† At that time the usual stipend of a university lecturer was £50 a year; Cobbett received £200.

several years spent his winter vacations in Switzerland. He was also an enthusiastic rider of the "ordinary" or "penny-farthing" bicycle before the introduction of the "safety" bicycle. The "ordinary" was a distinctly dangerous machine built to suit the height of the rider, the handle bars, placed a few inches above the very large front wheel, being at the height of the rider's chin, and the saddle, on the "back-bone" connecting the large front and the small back wheel, almost as high. The cranks were attached directly to the axle of the front wheel, so that this wheel made one revolution with each revolution of the pedal. The single brake consisted of a rubber block which could be pushed down against the narrow solid rubber tyre, a procedure which, unless carefully performed, was likely to cause an accident. At that time the roads were full of ruts and pot-holes which were liable to cause a spill if the back wheel went into them. In going downhill it was not an uncommon practice for the rider to place his legs over the handle bars so that his feet projected in front. Cobbett, long after he had taken to the "safety", used to relate how pleasant it was to ride on an "ordinary" because he could see the view over the hedges and walls and because going down a moderate slope with the wind behind was the nearest approach to flying that a human being was ever likely to achieve. He used to give graphic descriptions of some of his adventures on such a machine and especially of one when, by mistake, he rode down a long, steep and winding hill in Surrey. Once started on the steep slope it was impossible to stop, the speed became terrifying and rounding some of the curves the machine seemed to be nearly horizontal. He recalled his sensation of being very insecurely perched on a seat five feet above the road, his legs, projecting over the handle bars, increasing the difficulty of steering, the pedals revolving so fast that any attempt to place his feet on them meant instant propulsion over the handles; any application of the brakes meant the same result and no hope for a safe termination until an upgrade or long flat stretch of road was reached.

After he came back to Cambridge in 1893, he explored all the surrounding country-side on his cycle, taking special interest in prehistoric earthworks, evidences of "common-field" cultivation, terraces and old churches, many of which he photographed. On one of these excursions, about 1910, he went to the lonely village of Strethall to ascertain for himself what memory remained of a remarkable occurrence there about 60 years previously. In March 1849 the squire, Nehemiah Perry, shot dead a notorious Gipsy poacher, one Abraham Green, who, with others, had broken into his house at night with intent to murder him. At the inquest held next day a verdict of justifiable homicide was brought in, and the squire was thanked by the foreman on behalf of the neighbourhood and congratulated by the coroner. The body was then exhibited for several days in the belfry of the church at 3d. a head and, for various reasons,

" hundreds came to see it from all parts of the country ". When it became undesirable to keep it there any longer, Perry would have liked to have " nailed it up on his barn with the hawks and hand-saws " but, being persuaded that this procedure was no longer possible, doubled it up in a large game hamper and sent it to his friend, Sir George Paget, regius professor of physic, with the following letter on top of it.

Dear Mr Paget,

I have shot a man !

N. Perry.

Perry never afterwards " went out-of-doors without his gun, not even across his garden ".

Cobbett, finding no one in the house, asked an old gardener if he had heard of the tale and if it was true. The old man said " Yes, Maister, it be true. Little Abel's body, they do say, it be in the Moosum at Cambridge, but his inards, they be under that there stone ", and added after a pause " I put them there myself ". Green's sternum, the lower part perforated by 10 shot holes, is in the pathology museum at Cambridge.*

About 1910 Cobbett bought his first motor car and after that his excursions were much extended. He was particularly happy in taking out his friends or boys in whom he was interested, including junior laboratory assistants, to see the country and the objects about which he was so enthusiastic, and very often to swim in the rivers. On one occasion, when he was well over 70, he went to assist another small party which was being roughly hustled off the river bank by a farmer and his men and received a thrust in his chest with a pitch-fork, fortunately without serious consequences.

Before the second world war, Cobbett went on several Hellenic cruises and became greatly interested in Palestine, visiting most of the places mentioned in the historical books of the Old Testament. He thought of completing and publishing his observations, but the outbreak of war prevented this project.

About 1912 he acquired Inch-ma-home, in Adams Road, and lived there for the rest of his life.

Sir Cyril Fox, Director of the National Museum of Wales, who knew Cobbett intimately for many years of his long life, has kindly supplied the following appreciation.

" In his middle age (when I first met him he was about 40) he was, I thought, a lovely person, with a most winning smile and beautifully proportioned, with small hands and feet. His interest in young people was well known in Cambridge, and he helped to pay for the education, up to the University, of more than one. My first meeting

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* A very interesting account of this occurrence is given by G. Wherry (Camb. Antiquarian Soc. Communications, 1905, xi, 269), showing the great changes in customs, beliefs and procedures between 1850 and 1900.

with him at the age of 18 or 19 may be cited as characteristic. I was sitting on the sea front at Worthing (where I was being taught— of all things !—the art and mystery of market gardening under glass) when he entered into conversation with me, asking about the antiquities of the district. I showed him a scale plan I had made, and happened to have in my bicycle basket, of Cisbury hill-foot, and on the next Saturday afternoon we went there together. An invitation to stay with him at Round Church Street followed, and when, shortly after- wards, he became one of the pathologists to the Royal Commission on Tuberculosis and that body wanted a clerk, he offered me the job. Being thus for the first time brought into direct and continuous contact with University people and scientific humanism, I feel I owe my happy life of congenial work mainly to him.

   " He several times told me that he took up bacteriology because, when he had completed his medical training, he became independent through the death of his father, and determined to do some of the unpaid work of the world. The independence of outlook which the possession of an unearned competence can give was characteristic of him. He had no illusions and allowed none to relatives or friends. Though very fond of his sister Effie, he used to make fun of her attitude to life and people. A good example of his outspokenness occurred after I left Cambridge in 1924. In a couple of years time I returned to stay with him. On that occasion he remarked ' You know, Fox, I always cite you as the best example I know of a man made by the War. Your generation, in your field of interest (as in others), was mostly killed in the War, so there was no competition for vacant posts when it ended, and you just walked in where you liked '.

   " Cobbett was a delightful companion, treating youngsters as though they were his equals and laying before them the riches of his mind without stint. He taught me by inference and indirectly the importance of exact statement and to avoid emotionalism in expression. He was most generous ; never had I to pay when we were together, whether it was a meal or a week-end at a pub. He liked good food, but professed no interest in or knowledge of wine ; bottled beer was the staple drink at his table.

   " He took an immense interest in gardening and delighted to show friends the beauties and variety of his rock- and water-garden. In his own house he was socially superb. For years I was welcome at any time, at any meal ; but most welcome in the evenings. I would find him reading in his armchair beside the revolving bookcase, smoking his favourite Craven in the pink tin. Sister Effie would be on the other side of the fire. He would look up, smile and pass the tin, and perhaps invite my attention to the latest literature in the bookcase or listen to any remarks I might make ; and then continue his reading, stopping from time to time to discuss any point that interested him or to listen to anything I had to say. Thus there was never that awkward pause which so commonly occurs in ordinary households

when, one's host having directed his whole time to the visitor and topics of mutual interest being exhausted, the latter realises that he had better rise and depart.

"Cobbett had the educated and travelled Englishman's love of furniture and decorative porcelain, particularly Chinese of the later dynasties, but he had no particular knowledge of either and very sensibly only collected enough to furnish his rooms as he liked. His interest in pictures was mainly, I think, the result of holidays in Italy. Gothic architecture, particularly church architecture, interested him greatly and I owe my interest therein mainly to his teaching. His archæological and architectural leanings were just part of the good life as he conceived it, giving point and interest to his expeditions into the Cambridgeshire countryside which he so dearly loved and to the exploration of any district in which he was holiday-making. He never, so far as I know, made any claims to professional competence in either subject or joined any archæological society other than the *Cambridge Antiquarian Society, in the affairs of which he took great* interest. But, as one would expect, his few printed papers in this field were sound pieces of research, effectively set out.

"Cobbett was, as I have shown, a friendly, lovable man, meeting gentle and simple, young and old, as equals. He was an agnostic, but in his dealings with his fellow-men, in my experience, observed the spirit of Christ. Consequently he was loved by his nephews and nieces and well served by his dependents. In this latter category I particularly recall his gardener and his wife: "George" became his body servant in the last sad years of his long life, when he wrote to me more than once 'I have lost interest in everything; am always bored, with nothing to do '".

Cobbett was an original member of the Pathological Society, but for some years had been a very infrequent attender at its meetings.

G. S. GRAHAM-SMITH

BIBLIOGRAPHY

SCIENTIFIC PUBLICATIONS

1894-96

L. COBBETT and W. S. MELSOME. On local and general immunity. This *Journal*, 1894-96, iii, 39-69.
Contribution to the study of the serum therapeutics of diphtheria: an experimental inquiry into the relative merits of several methods of immunising horses with the object of obtaining from them a remedy for this disease. This *Journal*, 1894-96, iii, 327-343.

1896-97

L. COBBETT and G. C. PHILLIPS. The pseudo-diphtheria bacillus. This *Journal*. 1896-97, iv, 193-205.

1897

Contribution à l'étude de la physiologie du bacille diphtérique   *Ann Inst Pasteur*, 1897, xi, 251 263

Shock and collapse   *In* Allbutt's System of Medicine, *London*, 1897, vol iii, pp 320 332

1898

Alkalised serum as a culture medium for the bacterial diagnosis of diphtheria   *Lancet*, 1898, i, 362

Anti streptococcic serum   *Lancet*, 1898, i, 986 992

The result of the treatment of diphtheria by antitoxin in London compared with that in Paris and Berlin   *Lancet*, 1898, ii, 1457 1461

L COBBETT and W S MELSOME   Ueber den directen Einfluss der Entzundung auf die locale Widerstandsfahigkeit der Gewebe gegenuber der Infection   *Cbl allg Path*, 1898, ix, 829 838

Alkalisiertes Rinder und Pferdeserum als Hilfsmittel bei der Diphtherie diagnose   *Cbl Bakt*, 1898, xxiii, I Abt, 395 397

L COBBETT and A A KANTHACK   Ueber das Schicksal des Diphtherietoxins im Tierorganismus   *Cbl Bakt*, 1898, xxiv, I Abt, 129 137

Der Einfluss des Filtrierens auf das Diphtherie Antitoxin   *Cbl Bakt*, 1898, xxiv, I Abt, 386 391, 415 419

1899

The resistance of rats to diphtheria toxin   *Brit Med J*, 1899, i, 902 903

The origin of antitoxin   is it present in the blood of some normal animals ?   *Lancet*, 1899, ii, 332 337

A preliminary note on the occasional toxic action of horse serum on the guinea pig   *J Physiol*, 1899, xxiv, xxix xxxi

Enthalt das normale Pferdeserum Diphtherieantitoxin ?   *Cbl Bakt*, 1899, xxvi, I Abt, 548 554

1899 1900

The nature of the action of antitoxins   This *Journal*, 1899 1900, vi, 193 219

1900

L COBBETT and J H C DALTON   Some experiments on the sterilising properties of unconfined superheated steam   *Lancet*, 1900, i, 299 303

Diphtheria in the horse   *Lancet*, 1900, ii, 573 574

Has antitoxine reduced the death rate from diphtheria in our large towns ?   *Edinb Med J*, 1900, vii, 521 547

Atmo thermo steriliser   *J Physic Therap*, 1900, i, 39 40

1901

G H F NUTTALL, L COBBETT and T STRANGEWAYS PIGG   Studies in relation to malaria   I The geographical distribution of *Anopheles* in relation to the former distribution of ague in England   *J Hyg*, Camb, 1901, i, 4 44

An outbreak of diphtheria checked by prophylactic use of antitoxin, and the isolation of infected persons   *J Hyg*, Camb, 1901, i, 228 234

The result of 950 bacteriological examinations for diphtheria bacilli during an outbreak of diphtheria at Cambridge and Chesterton   *J Hyg*, Camb, 1901, i, 235 259

Observations on the recurrence of diphtheria in Cambridge in the Spring of 1901   *J. Hyg*, Camb, 1901, i, 485 499

1902

L. COBBETT and G. S. GRAHAM-SMITH. Report on the measures taken to check an outbreak of diphtheria in Colchester in 1901, *Colchester.*

1904-05

The progress and prevention of diphtheria. *J. Roy. San. Inst.,* 1904-05, xxv, 405-411.

1907

The pathogenic effects of human viruses. Royal Commission on Human and Animal Tuberculosis, 1907, pt. ii, appendix, vol. ii, pp. 1-1217.

The cultural characters of the tubercle bacilli obtained from man. *Ibid.,* 1907, pt. ii, appendix, vol. iii, pp. 21-25.

Report on the stability of virulence of the tubercle bacillus of human origin in artificial culture, including the influence of glycerin in culture media. *Ibid.,* 1907, pt. ii, appendix, vol. iii, pp. 185-209.

Report on the minute non-progressive tubercles found in calves injected with tubercle bacilli of human origin which are unable to provoke a progressive infection in this species of animal. *Ibid.,* 1907, pt. ii, appendix, vol. iii, pp. 211-218.

Report on the distribution of non-virulent tubercle bacilli of human origin in the body of the calf after subcutaneous injection. *Ibid.,* 1907, pt. ii, appendix, vol. iii, pp. 219-233.

Report on the influence of individual susceptibility and of dose on the *severity* of an experimental infection by tubercle bacilli of human origin. *Ibid.,* 1907, pt. ii, appendix, vol. iii, pp. 247-258.

Report on congenital tuberculosis at Blythwood Farm. *Ibid.,* 1907, pt. ii, appendix, vol. iii, pp. 259-261.

1909

Portals of entry of tubercle bacilli into the body. *Brit. Med. J.,* 1909, ii, 867-868.

1909-10

The portals of entry of the tubercle bacilli which cause phthisis. This *Journal,* 1909-10, xiv, 563-604.

1910

The progress of infection in phthisis. *Lancet,* 1910, ii, 1539-1542.

L. COBBETT and G. S. GRAHAM-SMITH. An *investigation of the* pathology of " grouse disease ". *J. Hyg.,* Camb., 1910, x, 1-36.

1910-11

Introductory address to a discussion on *portals of entry in phthisis. Proc.* *Roy. Soc. Med.,* 1910-11, iv, Sect. Stud. Dis. Childr., 205-219, 232-234.

1911

L. COBBETT and G. S. GRAHAM-SMITH. Grouse disease—pathology. *In The* grouse in health and disease. *London,* 1911, vol. i, pp. 273-307.

The causation of tuberculosis. *Cambridge,* 1911, pp. 39.

L. COBBETT and G. S. GRAHAM-SMITH. The passage of bacteria from the mouth to the lung. *Proc. Camb. Phil. Soc.,* 1911, xvi, 125-138.

1913

Two cases of spontaneous tuberculosis in the rabbit caused by the *avian* tubercle bacillus. *J. Comp. Path. and Therap.,* 1913, xxvi, 33-45.

1917

Wassermann's reaction *Practitioner*, 1917, xcix, 72 84

The causes of tuberculosis, together with some account of the prevalence and
  distribution of the disease *London*, 1917, pp 707 , *New York*, 1917,
  pp 723

1918

An acid fast bacillus obtained from a pustular eruption *Brit Med J* , 1918,
  ii, 158 159

The " influence of dose ", and its bearing on the question of the communicability
  of tuberculosis *Practitioner*, 1918, c, 402 415

Tuberculosis and the war , " prevention is better than cure " *Brit J Tuberc* ,
  1918, xii, 16 19

1920

Will tuberculosis die out ? *Discovery, Lond* , 1920, i, 146 151

1922

The rôle of the three types of tubercle bacilli in human and animal tuberculosis.
  Lancet, 1922, i, 979 983 , *J State Med* , 1922, xxx, 160 176

1923

L Cobbett and D Keilin Mucoid casts from the vermiform appendix This
  Journal, 1923, xxvi, 297 299

1923 24

Vegetable decomposition in ditch water simulating sewage contamination
  J Hyg , Camb , 1923 24, xxii, 389 396

1924 25

Tuberculosis in man and domesticated animals The relative susceptibility to the
  disease of their various organs *Tubercle*, 1924 25, vi, 1 17

The resistance of civilized man to tuberculosis , is it racial or individual in origin ?
  Tubercle, 1924 25, vi, 577 590

1925

Decline in the death rate from tuberculosis *New York Tuberculosis and Health
  Assoc Bull* , 1925, vi, 10 15

1926

The type of tubercle bacillus found in tuberculosis of the cat *J Comp Path.
  and Therap* , 1926 xxxix, 142 148

1928

The type of tubercle bacillus commonly present in tuberculous lesions in bones
  and joints *Brit Med J* , 1928, i, 626 627

1930

The decline of tuberculosis and the increase in its mortality during the war.
  J Hyg , Camb , 1930, xxx, 79 103

1931–32

The discovery of the tubercle bacillus by Robert Koch in 1882 and the state of
  bacteriology at the time *Camb Univ Med Soc Mag* , 1931 32, ix, 93 102

1932

The rabbit as a means of distinguishing the human from the bovine type of tubercle bacillus : the lesions caused in the rabbit by tubercle bacilli of the human type.   This *Journal*, 1932, xxxv, 681-699.

1933

The decline in the death rate of diphtheria compared with that of scarlet fever. *Brit. Med. J.*, 1933, ii, 139-140.

1933-34

Louis Pasteur, the man and pioneer.   *Camb. Univ. Med. Soc. Mag.*, 1933-34, xi, 65-74; 147-157.

PUBLICATIONS ON ARCHÆOLOGICAL SUBJECTS

L. COBBETT and C. F. Fox.   The Saxon Church of Great Paxton, Hunts. *Camb. Antiq. Soc. Proc.*, 1924, xxv, 50-77.

The Church of St. John, Duxford, Cambs.   *Cambs. Chronicle*, 1926, 19th May.

Windows inserted in the tower of St Benet's Church, Cambridge, in 1586. *Camb. Antiq. Soc. Proc.*, 1927, xxviii, 83-90.

Ickleton Church in the XVIII Century. *Camb. Antiq. Soc. Proc.*, 1929, xxx, 29-53.

The Tympanum of St John's Church, Duxford, Cambs. *Camb. Antiq. Soc. Proc.*, 1929, xxx, 63-77.

Saxon Grave-stones. *Cambs. Chronicle*, 1939, 25th Nov.

Saxon Carvings found at Ely. *Antiq. J.*, 1934, xiv, 62:

Grouse from the Nile or the truth about the plagues of Egypt. *Camb. Rev.*, 1934-35, lvi, 7.

L. COBBETT and W. M. PALMER.   The Hospitals of St John and St Mary Magdalene, Ely, and the remains of Gothic buildings still to be seen there at St John's Farm. *Camb. Antiq. Soc. Proc.*, 1936, xxxvi, 58-108.

Ornament in Hadstock Church, Essex. *Camb. Antiq. Soc. Proc.*, 1937, xxxvii, 43-46.

L. COBBETT and E. SAVILLE PECK.   Stone bowl found on the Guildhall site. *Camb. Antiq. Soc. Proc.*, 1937, xxxvii, 47-49.

Notes on Ickleton Church.   1938 (privately printed).

Sydney Arthur Monckton Copeman
(Born 21st February 1862.   Died 11th April 1947)

(PLATE CXII)

WITH the death of Monckton Copeman there passed one who had done much to maintain and enhance the high standard of English state medicine.   The eldest son of the Rev. Canon A. C. Copeman, he was born at Norwich on 21st February 1862, and was educated at King Edward VI's School, Norwich, and Corpus Christi College, Cambridge, where he obtained a second class in the Natural Sciences Tripos in 1882.   He went to St Thomas's Hospital for his medical training, took the M.B. Cambridge in 1886 and proceeded to the M.D. in 1890.

PLATE CXII

S Monckton Copeman. 1931.

He was elected F.R.C.P. in 1899. For a short period after qualification he was assistant lecturer in physiology and morbid histology at St Thomas's, and in 1891 he was appointed a medical inspector of the Local Government Board, a post which he held until 1919, when he became a medical officer of the Ministry of Health. He was for many years lecturer in public health at Westminster Hospital and in 1916-17 he was Colonel-in-Charge of the Hygiene Department of the Royal Army Medical College. He retired from the service of the Ministry of Health in 1925.

Copeman's earlier scientific papers, dating from the St Thomas's period, were mainly concerned with the blood and blood diseases. While this work is not so well known as his later researches, this period, when he was associated with Sherrington and others, must have provided a valuable training in the experimental method of approach to medical problems and probably had a profound influence on his later activities. In 1891 he described a simple method of determining the specific gravity of the blood by observing the behaviour of drops of blood added to mixtures of glycerine and water of known specific gravity. This technique was later used in an extensive investigation carried out in collaboration with Sherrington on alterations in the blood produced by injections of saline or water, repeated bleeding and other procedures, some of which induced shock in the experimental animals.

When Copeman became medical inspector to the Local Government Board he commenced the work on smallpox and vaccination on which his scientific reputation chiefly rests. The results of his observations were published in numerous papers and communicated to various societies between 1891 and 1904. Following on his study of the bacteriology of vaccine lymph in 1891, he published his important observations on the effect of glycerine in destroying extraneous bacteria in lymph while leaving the specific infective agent undamaged. The addition of glycerine to vaccine lymph had been practised by others, but it would seem that the selective bactericidal action of the added glycerine was first clearly stated by Copeman. In his paper published in 1893, however, he quotes from a paper published in 1892 by Sclavo of Rome, who had recorded somewhat similar effects. Further studies in this field were published jointly with Blaxall in 1896, showing the inhibitory effect of glycerine added to pure cultures of various bacteria in peptone broth and kept in the cold and at room temperature. They found, on the other hand, that soft paraffin and lanoline, which had been added by some workers to vaccine lymph, had no inhibitory effect on contaminating bacteria. Copeman was an ardent advocate of the use of glycerinated calf lymph for vaccination against smallpox, and the methods which he detailed in his Milroy lectures in 1898 for the preparation of vaccine lymph have been in use in this country for many years. Of this aspect of his work Dr Mervyn Gordon writes :—" The glycerination of calf

lymph was a great advance, because it rendered vaccination both safer and more sure. It was Copeman's Milroy Lectures that provided the evidence on which the Central Health Department was able to induce the Treasury to found and support the Government Lymph Department (G.L.E.). The high and consistent standard of potency achieved by Blaxall and his successors in the quality of the lymph would not have come about without this preliminary work of Copeman's ".

Copeman was also greatly interested in the relationship of vaccinia to variola, a subject which had been a matter of controversy since the work of Jenner a century before. In his first attempts to repeat the observations of others on the inoculation of calves with material derived from cases of smallpox, Copeman apparently succeeded in one out of four experiments, and, after several transfers in the skin of calves, typical vesiculation resulted. These experiments were made at the Government Lymph Establishment in Conduit Street: later, he admitted (1896) the advisability of repeating the experiments at an institution where no work with vaccinia was being carried on. (In 1903 he recorded that four attempts, made in 1901, to variolate calves directly were unsuccessful, although good strains of lymph for human use were obtained by passing the same material first on monkeys and then to calves.) In 1893 he published his first experiments on variola and vaccinia in monkeys and a detailed account of these results appeared in the second volume of this *Journal* (1893-94). He showed that variolation of monkeys could readily be effected, that monkeys so treated were thereafter immune to vaccinia and that similarly vaccinated monkeys became immune to variolous material. The variolous infection in monkeys was successfully transferred to calves and, after several passages in calves, vesicle fluid was used for the successful vaccination of many children. As a result of his own experiments and the recorded observations of others Copeman concluded that smallpox and cowpox were descended from a common stock which resembled vaccinia more than it resembled smallpox.

He published observations on the presence of a small bacillus in vaccine and variola lymph and, although he was unable to cultivate the organism on ordinary culture media, he claimed to have succeeded in vaccinating children with cultures of human variola material which had been incubated in eggs for a month. One can only suggest that this result may have been due to survival of virus under these conditions or to contamination of his material with vaccine virus. Copeman was unwilling to believe that the bodies described by Guarnieri represented the infective agent of variola or vaccinia. Most of his work on variola and vaccinia was incorporated in the Milroy Lectures of 1898 and published in his book, *Vaccination, its natural history and pathology*, the following year. This work established his scientific reputation and led to his election as a Fellow of the Royal Society in 1903. Later he became a member of the Ministry of Health's Depart-

mental Committee on Cancer and wrote several papers on this subject. The report which he published with Greenwood in 1926, on observations into the incidence of cancer among certain religious orders, failed to substantiate the notion that absence of meat from the diet greatly lessened the incidence of this disease.

Copeman was enthusiastic about the value of immunisation in the prevention of infective disease and was a pioneer in immunisation against diphtheria in Great Britain. He was impressed by the American work on diphtheria and scarlet fever, although he appreciated that immunisation against scarlet fever was not so readily effected as against diphtheria. These matters and the serum prophylaxis of measles were the subject of his presidential address before the Epidemiological Section of the Royal Society of Medicine in 1926. Copeman's scientific interests, however, were wide and the range of subjects covered by his writings is shown by the titles in the appended bibliography.

Sir Arthur MacNalty writes of him :—" His duties as a medical inspector of the Local Government Board and, subsequently, as a medical officer of the Ministry of Health entailed much administrative work for the Central Health Department both at home and abroad. He acted as Government delegate on many occasions and served on many departmental committees. But his real interest lay in scientific research, and his alert mind, fertile in new ideas, was directed to numerous subjects of investigation. He took an active part in the work of many medical societies and organisations. He was a keen naturalist and biologist, and, as a member of the Zoological Society, did much to develop its work on scientific lines ". He adds, on a more personal note, " Monckton Copeman was tall and of distinguished appearance, with clear-cut features. In speech and gesture he was animated and spoke well and fluently. He had a good literary style and a gift of exposition which adorn his many scientific writings. For several years at the Ministry of Health we shared a room together, and it was then that I learned to appreciate how kind and encouraging he could be to a younger colleague. Although occupied with much responsible work, he always found time to listen to other men's problems and to advise them out of his long experience in official and scientific investigation. He was well read, highly cultured and a delightful companion. He had a happy family life, and those who enjoyed Dr and Mrs Copeman's hospitality at Regent's Park or at Hampstead will remember the gracious hostess who was so keenly interested in her husband's many avocations ".

In addition to his election to the Royal Society in 1903 Copeman received many distinctions, for example, the Cameron prize of the University of Edinburgh (1899), the Fothergill gold medal of the Medical Society of London (1899), the Buchanan gold medal of the Royal Society (1902), the Jenner medal of the Royal Society of Medicine (1925) and the gold medal of the International Faculty of

Sciences (1938). In 1913 he was appointed a Knight of Grace of the Order of St John of Jerusalem. He was an original member of the Pathological Society and for many years a regular attender at its meetings.

After his official retirement in 1925 Copeman interested himself in local government. He was a member of the London County Council and chairman of their Hospitals and Medical Services Committee, and he was chairman of the Public Health Committee of the Hampstead Borough Council.

He married Ethel Margaret, youngest daughter of the late Sir William Boord, Bart., and had a son and two daughters.

A. W. DOWNIE

BIBLIOGRAPHY

1887

The blood in pernicious anæmia. *Lancet*, 1887, i, 1076-1079 ; and *St Thomas's Hosp. Rep.* (1886), 1887, n.s. xvi, 155-171.

1889

Report on the medico-legal detection of human blood. *Brit. Med. J.*, 1889, ii, 190-193.

Unique case of biliary fistula, with some observations on the bile obtained from it. *Lancet*, 1889, i, 1026-1027 ; 1075-1077.

J. S. BRISTOWE and S. A. M. COPEMAN. A case of paroxysmal hæmoglobinuria with experimental observations and remarks. *Lancet*, 1889, ii, 256-259 ; 307-309 ; and *Proc. Med. Soc. Lond.*, 1889, xii, 256-272.

S. A. M. COPEMAN and W. B. WINSTON. Observations on human bile obtained from a case of biliary fistula. *J. Physiol.*, 1889, x, 213-231.

1890

Contribution to a discussion on lead poisoning from drinking water. *Brit. Med. J.*, 1890, ii, 498.

Note on an improvement in the use of the hæmocytometer. *Lancet*, 1890, i, 73-74.

The crystallization of hæmoglobin in man and the lower animals, and of hæmochromogen in man. *J. Physiol.*, 1890, xi, 401-409.

S. A. M. COPEMAN and C. S. SHERRINGTON. A method intended to determine the quantity of blood in a living animal. *J. Physiol.*, 1890, xi, viii.

Myohæmatin. *J. Physiol.*, 1890, xi, xxii.

The pathology of paroxysmal hæmoglobinuria : an experimental research. *Practitioner*, 1890, xlv, 161-183.

The medico-legal detection of human blood. *St Thomas's Hosp. Rep.* (1888), 1890, n.s. xviii, 95-116.

1891

Report on the specific gravity of the blood in disease. *Brit. Med. J.*, 1891, i, 161-163 ; 448.

Hæmatoporphyrin in the urine. *Trans. Path. Soc. Lond.* (1890-91), 1891, xlii, 364-371.

1891-92

Bacteriology of vaccine lymph. *Trans. Epidemiol. Soc. Lond.*, 1891-92, n.s., xi, 70-80.

1892

The bacteriology of vaccine lymph. *Trans. VIIth Internat. Cong. Hyg. and Demogr.*, London (1891), 1892, ii, 319-326.
The influence of soil on health. *In* Stevenson and Murphy's Treatise of hygiene, 1892, vol. i, pp. 307-390.

1892-93

Variola and vaccinia: their manifestations and inter-relations in the lower animals. *Trans. Epidemiol. Soc. Lond.*, 1892-93, n.s., xii, 142-166.

1893

The bacteriology of vaccine lymph, with special reference to an improved method for its storage and preservation. *Brit. Med. J.*, 1893, i, 1256-1258.
C. S. SHERRINGTON and S. A. M. COPEMAN. Variations experimentally produced in the specific gravity of the blood. *J. Physiol.*, 1893, xiv, 52-96.
Experiments on variola and vaccinia. *Proc. Roy. Soc.*, 1893, liv, 187-189.
The inter-relations and bacteriology of variola and vaccinia. *Med. Press and Circ.*, 1893, n.s., lv, 610.
On an outbreak of scarlatina at Bush Hill Park, in the Enfield and Edmonton urban sanitary districts. *Rep. Med. Off. Loc. Govt. Bd.* (1891-92), 1893, xxi, 69-78.

1893-94

Variola and vaccinia; their manifestations and inter-relations in the lower animals: a comparative study. This *Journal*, 1893-94, ii, 407-427.

1894

Opening paper of a discussion on the pathology of vaccinia. *Brit. Med. J.*, 1894, ii, 631-634.
Report on an outbreak of typhus fever in the Wigan and Ince-in-Makerfield urban sanitary districts. *Rep. Med. Off. Loc. Govt. Bd.* (1892-93), 1894, xxii, 83-100.
Report on an outbreak of epidemic skin disease. *Rep. Med. Off. Loc. Govt. Bd.* (1893-94), 1894, xxiii, 164-279.

1894 95

The pathology of vaccinia. *Publ. Hlth.*, 1894-95, vii, 108-111.

1895

Antitoxins and other organic remedies. *Brit. Med. J.*, 1895, ii, 835-836.

1895 96

The influence of subsoil water on health. *San. Rec.*, Lond., 1895-96, n.s., xvii, 666-663.
The discovery of the variola bacillus. *San. Rec*, Lond, 1895 96, n.s., xvii, 699-700.

1896

Pathology of vaccinia and variola. *Brit. Med. J.*, 1896, i, 7-10.
The bacteriology of vaccinia and variola. *Brit. Med. J.*, 1896, i, 1277-1279.
The influence of subsoil-water on health. *J. Roy. San. Inst.*, 1896, xvii, 23-42; and *Lancet*, 1896, ii, 166 168.
Small-pox and vaccinia: their manifestations and inter-relations in man and the lower animals. *Practitioner*, 1896, lvi, 459-480.

The influence of subsoil water on health. *Med. Times and Hosp. Gaz.*, 1896, xxiv, 129-130; 145-146; 177-178.

Report on an outbreak of enteric fever at Loddon, in the rural district of Loddon and Clavering. *Rep. Med. Off. Loc. Govt. Bd.* (1895-96), 1896, xxv, 63-72.

Report on an outbreak of epidemic skin disease in the Enfield Workhouse of the Edmonton Union. *Rep. Med. Off. Loc. Govt. Bd.* (1895-96), 1896, xxv, 93-100.

S. A. M. COPEMAN and F. R. BLAXALL. Reports on the influence of glycerine, of lanoline, and of vaseline, in inhibiting the growth of micro-organisms commonly found in vaccine lymph. *Rep. Med. Off. Loc. Govt. Bd.* (1895-96), 1896, xxv, 283-292.

1897

S. A. M. COPEMAN and F. R. BLAXALL. Experiments on the action of glycerine upon the growth of bacteria. *Rep. Brit. Assoc. Adv. Sci.* (1896), 1897, lxvi, 986.

Pathology of vaccinia. *In* Allbutt's System of medicine, London and New York, 1897, vol. ii, pp. 636-657.

Hæmoglobinuric fever. *In* Allbutt's System of medicine, London and New York, 1897, vol. ii, pp. 742-752.

1898

The Milroy Lectures on the natural history of vaccinia. *Brit. Med. J.*, 1898, i, 1185-1189; 1245-1250; 1312-1318; and *Lancet*, 1898, i, 1237-1243; 1303-1310; 1375-1382.

S. A. M. COPEMAN and F. R. BLAXALL. The action of glycerine on the tubercle bacillus. *Rep. Brit. Assoc. Adv. Sci.* (1897), 1898, lxvii, 829-830.

The clinical examination of the blood. *In* Allbutt's System of medicine, London and New York, 1898, vol. v, pp. 408-464.

Hæmoglobinuria. *In* Allbutt's System of medicine, London and New York, 1898, vol. v, pp. 621-635.

1899

S. A. M. COPEMAN and G. MANN. The histology of vaccinia. *Rep. Med. Off. Loc. Govt. Bd.* (1898-99), 1899, xxviii, 505-546.

Vaccination, its natural history and pathology. 1899, London, pp. 1-257.

1900

The micro-organism of distemper in the dog, and the production of a distemper vaccine. *Proc. Roy. Soc. Lond.*, 1900, lxvii, 459-461.

1901

On the general sanitary circumstances and administration of the borough of Boston. *Rep. Med. Off. Loc. Govt. Bd.* (1900-01), 1901, xxx, 77-95.

1902

Modern methods of vaccination and their scientific basis. *Med.-Chir. Trans.*, 1902, lxxxv, 243-270; and *Lancet*, 1901, ii, 1718-1723.

1902-03

The inter-relationship of variola and vaccinia. *Proc. Roy. Soc. Lond.*, 1902-03, lxxi, 121-133; and *Cbl. Bakt.*, I Abt., Ref., 1902-03, xxxii, 705-716.

1904

Report on the investigation of an outbreak of cow-pox on a farm at Buckland, near Reigate. *Rep. Med. Off. Loc. Govt. Bd.* (1902-03), 1904, xxxii, 258-266.
S. A. M. COPEMAN and F. G. PARSONS. Observations on the sex of mice. *Proc. Roy. Soc.*, 1904, lxxiii, 32-48.

1905

Report on an outbreak of epidemic skin disease at the Central London Sick Asylum, Hendon. *Rep. Med. Off. Loc. Govt. Bd.* (1903-04), 1905, xxxiii, 133-146.

1906

S. A. M. COPEMAN and H. W. HAKE. Note on determinations of the amount of physiologically active hydrochloric acid in the stomachs of normal mice and of mice suffering from cancer experimentally produced. *Lancet*, 1906, ii, 1276-1277.
Pathology of vaccinia. *In* Allbutt and Rolleston's System of medicine, second edition, *London* and *New York*, 1906, vol. ii, pt. I, pp. 746-766.
Report to the Local Government Board on an investigation into the general sanitary circumstances of the Langport rural district, with special reference to the occurrence there of outbreaks of diphtheria and small-pox. *London*, 1906.

1907

Report on an outbreak of enteric fever at Fulbourn Asylum, near Cambridge, with special reference to risk of pollution of underground water supplies by the sewage of the asylum. *Rep. Med. Off. Loc. Govt. Bd.* (1905-06), 1907, xxxv, 146-168.
The Guthrie Lecture on some recent research work on cancer. *Practitioner*, 1907, lxxix, 185-202.

1907-08

S. A. M. COPEMAN and H. W. HAKE. On the amount of physiologically active hydrochloric acid in the stomachs of normal mice, and of mice suffering from cancer experimentally produced. This *Journal*, 1907-08, xii, 127-128 (abstract).

1908

S. A. M. COPEMAN and H. W. HAKE. Report on a study of the variations in the secretion of hydrochloric acid in the gastric contents of mice and rats, as compared with the human subject, in cancer. *Proc. Roy. Soc. B*, 1908, lxxx, 444-462; and *3rd Scientific Rep. Imperial Cancer Research Fund*, 1908, 398-419.

1909

Inoculation methods for the prevention of diseases to which the soldier is liable. *J. Roy. Inst. Publ. Hlth.*, 1909, xvii, 577-595.
Memorandum by Dr Copeman on investigation into possible carriage of infection by flies. Suggested "plan of campaign" in urban districts. *Rep. to the Loc. Govt. Bd. on Publ. Hlth. and Med. Subjects*, 1909, n.s. no. xvi, pp. 1-4.
C. S. SHERRINGTON and S. A. M. COPEMAN. Body metabolism in cancer. *Rep. Brit. Assoc. Adv. Sci.* (1908), 1909, lxxviii, 489-492.

1910

On the general sanitary circumstances and administration of the Ely urban district. *Rep. to the Loc. Govt. Bd. on Publ. Hlth. and Med. Subjects*, 1910, n.s. nos. xxxviii, pp. 1-12, and xxxix, pp. 1-19.

Note as to work in hand, but not yet published; and as to proposed further work in reference to flies as carriers of infection. *Rep. to the Loc. Govt. Bd. on Publ. Hlth. and Med. Subjects*, 1910, n.s. no. xl, pp. 45-48.

1911

C. S. SHERRINGTON and S. A. M. COPEMAN. Body metabolism in cancer. *Rep. Brit. Assoc. Adv. Sci.* (1910), 1911, lxxx, 297-300.

On an outbreak of enteric fever in the borough of Eccles, Lancashire, due to the consumption of ice-cream. *Rep. to the Loc. Govt. Bd. on Publ. Hlth. and Med. Subjects*, 1911, n.s. no. li, pp. 1-18.

S. A. M. COPEMAN, F. M. HOWLETT and G. MERRIMAN. An experimental investigation on the range of flight of flies. *Rep. to the Loc. Govt. Bd. on Publ. Hlth. and Med. Subjects*, 1911, n.s. no. liii, pp. 1-9.

Vaccination. *In* The Encyclopædia Britannica, 11th ed., 1911, London, vol. xxvii, pp. 831-834.

1913

On an outbreak of enteric fever in the borough of Harwich and its neighbourhood. *Rep. to the Loc. Govt. Bd. on Publ. Hlth. and Med. Subjects* (1912-13), 1913, n.s. no. lxxxiii, pp. 1-18.

Hibernation of house-flies. *Rep. to the Loc. Govt. Bd. on Publ. Hlth. and Med. Subjects*, 1913, n.s. no. lxxxv, pp. 14-19.

1914

S. A. M. COPEMAN, S. R. BENNETT and H. W. HAKE. On the nature uses and manufacture of ferro-chrome and other ferro-alloys. *Rep. to the Loc. Govt. Bd. on Publ. Hlth. and Med. Subjects*, 1914, n.s. no. xciii, pp. 1-22.

S. A. M. COPEMAN and E. E. AUSTEN. Do house-flies hibernate? *Rep. to the Loc. Govt. Bd. on Publ. Hlth. and Med. Subjects*, 1914, n.s. no. cii, pp. 6-9.

1915

Note on a successful method for the extermination of vermin infesting troops. *Brit. Med. J.*, 1915, i, 247-248; and *Lancet*, 1915, i, 273-274.

1916

Prevention of fly-breeding in horse-manure. *Lancet*, 1916, i, 1182-1184.

1917

The prevention and arrest of certain epidemic diseases in war time. *Med. Press and Circ.*, 1917, ciii, 228-231; and *J. State Med.*, 1917, xxv, 103-115.

1918

Extracts from a report by S. Monckton Copeman on an obscure disease, Encephalitis lethargica, as observed in Birmingham and Leicester. *Rep. to the Loc. Govt. Bd. on Publ. Hlth. and Med. Subjects*, 1918, n.s. no. cxxi, pp. 66-68.

1919

Observations on a colony of burrowing bees (*Andrena pulva*) on Primrose Hill. *Proc. Zool. Soc.* (1918), 1919, 304-306.

1919 20

The relationship of small pox and alastrim *Proc Roy Soc Med*, 1919 20, xiii, Sect Epidemiol and State Med, 237 260, and in *1st Ann Rep of the Chief Med Off*, M of Health, 1920, 271 282

1920

Experiments on sex determination *Proc Zool Soc* (1919), 1920, 433 435

1921

A report on certain prophylactic measures against diphtheria, based on recent American work and on the investigation of an outbreak of diphtheria at the Southmead Infirmary, Bristol *Rep on Publ Hlth and Med Subjects*, M of Health, 1921, no x, pp 9 28

Note on the capture (in London) of a rare parasitic fly, *Hammomyia (Hylephila) unilineata* Zett, *Proc Zool Soc*, 1921, 31 34

1922

S A M COPEMAN, R A O BRIEN, A J EAGLETON and A T GLENNY Experiences with the Schick test and active immunisation against diphtheria *Brit J Exp Path*, 1922, iii, 42 50

1924 25

Immunization against scarlet fever *Proc Roy Soc Med*, 1924 25, xviii, Sect Epidemiol and State Med, 51 58

1925

Water cress and body metabolism *Pharm J*, 1925, cxv, 386 387

A report on infestation of the human subject by the nematode worm, *Ascaris lumbricoides Rep on Publ Hlth and Med Subjects*, M of Health, 1925, no xxxi, pp 1 20

1926

Immunisation in certain infectious diseases *J State Med*, 1926, xxxiv, 384 396

S A M COPEMAN and M GREENWOOD Diet and cancer with special reference to the incidence of cancer upon members of certain religious orders *Rep on Publ Hlth and Med Subjects*, M of Health, 1926, no xxxvi, pp 1 33

The relationship of small pox and vaccinia *The Nation's Health*, 1926 viii, 1 2

1926 27

Presidential Address on immunization against certain infectious diseases *Proc Roy Soc Med*, 1926 27, xx, 43 51

1928

A British Medical Association Lecture on "Immunization against diphtheria, scarlet fever, and measles" *Brit Med J*, 1928, i, 833 835

Inoperable cancer, with special reference to treatment by means of "activated" fluorescein *J State Med*, 1928, xxxvi 642 653

"Activated" fluorescein in the treatment of inoperable cancer Report of the International Conference on Cancer, 1928, *London*, pp 256 257

1929

S. A. M. COPEMAN, F. COKE and C. GOULDESBROUGH. "Activated" (irradiated) fluorescein in the treatment of cancer. *Brit. Med. J.*, 1929, ii, 233-236.

1931

Activated (irradiated) fluorescein in the treatment of cancer. *Brit. Med. J.*, 1931, i, 658-660.

1933-34

Contribution to a discussion on vaccination. *Proc. Roy. Soc. Med.*, 1933-34, xxvii, 877-881 ; 890-891.

1934

Destruction of living cells (protozoa) by invisible ultra-violet rays below 4000 Å.U. (all-quartz optical system employed). *J. State Med.*, 1934, xlii, 665-668.

John Oliver Wentworth Bland

(Born 6th October 1899.   Died 10th May 1946)

(PLATE CXIII)

POSSIBLY Bland was not very well known to many members of the Pathological Society. He was still young when he died and fully half of his research career had been spent in Egypt, so that he hardly had an opportunity of playing a prominent part in the life of the Society.   But those who, like myself, had the privilege of close association with him in work and friendship realise that by his untimely death pathology lost not only an accomplished and enthusiastic investigator but also a charming and cultured colleague. John Oliver Wentworth Bland, was born at Eltham in Kent in 1899. Both his parents were distinguished people. His father, Hubert Bland, a writer and journalist, was one of the founders of the Fabian Society and acted as its secretary until his death, and his mother, who wrote under the pen name of E. Nesbit, achieved a considerable reputation by her books for children, which, I believe, are still widely read. John was the youngest of their four children, but since there was a gap of thirteen years between him and the younger of his sisters, his upbringing was rather that of an only child, which possibly accounted for a certain precocity. He early developed an avidity for knowledge of all kinds and at the age of five he liked above all to have read to him books on biology and cell life. Much of his childhood was spent at Well Hall, Blackheath, and it was in Blackheath that he had his early schooling. His father was prejudiced against the public schools and when John was too old to remain in the kindergarten of Blackheath High School, he was sent to a small private school in the neighbourhood. However, when John was only fourteen his father died, and at the

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John Bland.

instigation of his brother and sisters the plans for his education were reversed and it was decided to send him to the City of London School. Unfortunately his early education had been so much neglected that he was unable to pass the entrance examination at the first attempt and it was only after nine months' coaching that he succeeded and was accepted. He did well at the City of London School, but there can be no doubt that the environment of his home was the dominant factor in his education. His parents kept open house for their many literary and artist friends. Bernard Shaw, H. G. Wells, Spencer Pryce, Oswald Barron and Brangwyn were frequent visitors to the Bland home, and since John adored his home and spent as much time there as possible, it is hardly surprising that its intellectual and cultured atmosphere should have played an important part in moulding his character and determining his outlook. It was from one of these visitors to the Bland household, Frederick Rolfe, that odd mixture of literary genius and adventurer, the self-styled Baron Corvo, that John Bland acquired his strikingly peculiar handwriting. The young boy was dazzled by this strange personality and when at the age of sixteen he found himself dissatisfied with his own caligraphy he decided to copy the striking script of his hero whose letters he had kept. And so well did he succeed that it was almost impossible to tell which was which. Bland had decided on medicine as a career and had elected to go to Cambridge, but when the time came for this move the first World War had supervened and instead of going to the university he was called-up for military service. In some ways Bland probably profited by this change. He was not retained very long in the army, for when his training in the Royal Engineers had just reached completion the war ended and he was demobilised. No doubt the experience of army life gave Bland that added maturity which enabled him better to profit by his university career than if he had gone there straight from school. When, however, he was finally free to go to Cambridge it was found that the family finances were insufficient to support this project, and it might have had to be abandoned had it not been for the generosity of Bernard Shaw who undertook to provide the money. So Bland went to Cambridge, and from there to St George's Hospital for his clinical training. There is no outstanding success to record in his undergraduate career ; in fact, although he worked satisfactorily, there was little indication of those potential qualities which his later research career was to reveal. He qualified by taking the L.M.S.S.A. in 1924 and having held the usual house appointments he returned to Cambridge for post-graduate study, dividing his time between the Department of Pathology and the Strangeways Laboratory. The training he then obtained in the technique of tissue culture was to prove of particular value in his subsequent researches. In 1927 he was awarded a Freedom Fellowship and he joined me at the London Hospital in the investigation of viruses which I had started there a year earlier. At first Bland

was fully occupied in acquiring the technique of this new subject, but I soon realised that I had found in him a colleague of very great qualities. He had a well-trained mind and his freshness of outlook and enthusiasm were exhilarating and contagious. Soon he was doing original work of real value. He made a study of vaccinia virus which had as its main objective the quantitative estimation of this virus in morbid material and the ascertainment of its particle size. This was an extension of the work which I had been doing with the virus of herpes and, as in the case of this virus, he was able to show that vaccinia virus was much larger than Levaditi and his colleagues had claimed and that it was probably within the range of visibility with the microscope. It must be remembered that this was nearly twenty years ago, when the size and visibility of these viruses was a matter of considerable dispute. This work formed the subject of the thesis which he submitted when taking the M.B. Cambridge in 1929. In the first three years of Bland's tenure of his Freedom Fellowship he also collaborated with me on various virus problems such as the supposed relationship between the viruses of herpes and vaccinia claimed by Gildemeister, a claim we were unable to confirm, and the possibility of demonstrating complement fixation with viruses and their antisera which, as we showed, had been wrongly held to be impossible. In 1930 he turned his attention to the investigation of an outbreak in adults of what was thought to be glandular fever and obtained evidence which suggested that the causal agent was probably a toxoplasm. As in all investigations of diseases due to toxoplasms, which rely on transmission to animals for the demonstration of the parasite, Bland's work was vitiated by the presence of toxoplasms in some of his normal animals, and doubt concerning the true interpretation of his findings, which he recognised and freely admitted, still remains. The discovery of psittacosis virus and the demonstration that this virus was a large one which could be stained and seen more readily than viruses previously studied, opened up a new field of virus research in the exploration of which I invited Bland's co-operation : and again he proved to be an invaluable colleague. In the study of the developmental cycle of this virus, which formed the subject of a series of joint communications, Bland was able to follow the growth of the virus in tissue culture and so to confirm previous observations, which had depended on the examination of preparations made from animals at different stages of infection. With the exception of a study of human gliomata in tissue culture which he made with Dorothy S. Russell, work on psittacosis virus occupied the remainder of Bland's stay at the London Hospital. It culminated in the making of a cinema film of the development of psittacosis virus, a joint piece of work in which the technical ingenuity of R. G. Canti played an important part. Bland obtained the M.D. Cambridge in 1935, with a thesis embodying his virus researches.

In 1937 Bland left the London to take up an appointment in the

Department of Pathology at St Bartholomew's Hospital. This post was not held for long and his work during its tenure was seriously interfered with by illness. He had time, however, in conjunction with C. F. Robinow, to study the growth of vaccinia virus in tissue culture, which gave some indication of a developmental cycle. Shortly before the outbreak of the recent war Bland accepted an invitation to fill the research post at the Giza Ophthalmic Memorial Hospital, Cairo, rendered vacant by the retirement of F. H. Stewart. Here the principal subject of study was trachoma, and it was hoped that Bland's experience with the related virus of psittacosis would help him in his approach to this difficult problem. Like others who have investigated trachoma he encountered difficulties, some of which were insuperable. He was able to infect monkeys and to transmit the infection to human volunteers, but both the baboon and the grivet monkey, though susceptible, proved unsuitable for work with this virus because of a tendency to spontaneous folliculitis of the conjunctiva. Also he was unable to induce trachoma virus to multiply in tissue culture. Faced with this impasse Bland, no doubt, was glad of the opportunity of alternative work which the war brought him. Whether this was so or not, the fact is that his war duties left him little time for research. He was given an administrative post in the A.R.P. service in Cairo and his help in the elucidation of problems presented by virus diseases in our troops in the Near East was frequently sought. Bland did all this with his usual enthusiasm and thoroughness. In 1944 a return of the old trouble, pulmonary tuberculosis, which had been responsible for his illness while he was at St Bartholomew's, obliged him to relinquish all work and undergo hospital treatment in Palestine. Returning to this country about the end of 1944, his treatment was continued for a few months at the Osler Sanatorium, Oxford, and subsequently at home in his cottage in Ashdown Forest. By the middle of 1945 he seemed sufficiently recovered to start again ; he looked well and, apart from shortness of breath, seemed to be in good health. Since he was advised to avoid the atmosphere of cities and to live and work in the country, he joined the staff of the Pathological Department of the Royal West Sussex Hospital at Chichester with the object of making himself fully proficient in clinical pathology and eventually taking a permanent post as a clinical pathologist. It was felt that it would be difficult for him to find research work in a suitable environment and he was not in the least dismayed at the prospect of earning his living by routine diagnostic pathology. Hardly had he started work at Chichester, however, than his health deteriorated and he was obliged to retire once more to his country home. Here, uncomplaining and always cheerful, he rested and watched with delight the coming of spring in that beautiful part of Sussex, and he wrote hopefully to me about returning to work. Early in May, however, he was taken acutely ill and he died in St Bartholomew's Hospital on 10th May 1946. By

Bland's illness and death, pathology, and in particular virus research, has lost a valuable man. His work throughout was characterised by meticulous care, considerable technical skill and ingenuity, great enthusiasm and fundamental honesty; I have rarely met a research worker who kept such careful records of his experiments. His one defect was that, when left on his own, his research activity tended to flag. It was not that he was lacking in ideas or originality, but more than most people he required the stimulus provided by working in close association with others to maintain his enthusiasm and activity. In addition to being a talented research worker Bland was a delightful companion; his fresh and vivid mind, his interest in the arts and his love of the countryside all combined to make him so. It is sad to reflect that this cultured unassuming person is no longer with us.

Bland married Mrs Maud Salzman in 1935.

S. P. BEDSON

BIBLIOGRAPHY

1928

The suitability of the guinea-pig for experimental work with vaccinia virus. *Brit. J. Exp. Path.*, 1928, ix, 97-100.

S. P. BEDSON and J. O. W. BLAND. On the supposed relationship between the viruses of herpes febrilis and vaccinia. *Brit. J. Exp. Path.*, 1928, ix, 174-178.

Filter and centrifuge experiments with guinea-pig vaccinia virus. *Brit. J. Exp. Path.*, 1928, ix, 283-290.

1929

S. P. BEDSON and J. O. W. BLAND. A simple method for determining the electrical charge carried by virus particles. *Brit. J. Exp. Path.*, 1929, x, 67-70.

Filter and centrifuge experiments with neurovaccine. *Brit. J. Exp. Path.*, 1929, x, 145-150.

S. P. BEDSON and J. O. W. BLAND. Complement-fixation with filterable viruses and their antisera. *Brit. J. Exp. Path.*, 1929, x, 393-404.

1930

Glandular fever. An experimental investigation. *Lancet*, 1930, ii, 521-524.

1931

Glandular fever II. The protozoal nature of the experimental disease. *Brit. J. Exp. Path.*, 1931, xii, 311-319.

1932

S. P. BEDSON and J. O. W. BLAND. A morphological study of psittacosis virus, with the description of a developmental cycle. *Brit. J. Exp. Path.*, 1932, xiii, 461-466.

Immunisation with inactive vaccinia virus. *J. Hyg.*, Camb., 1932, xxxii, 55-68.

1933

Dorothy S. Russell and J. O. W. Bland. A study of gliomas by the method of tissue culture This *Journal*, 1933, xxxvi, 273-283.

1934

Dorothy S. Russell and J. O. W. Bland. Further notes on the tissue culture of gliomas with special reference to Bailey's spongioblastoma. This *Journal*, 1934, xxxix, 375 380.

S. P. Bedson and J. O. W. Bland. The developmental forms of psittacosis virus. *Brit. J. Exp. Path.*, 1934, xv, 243 247.

1935

J. O. W. Bland and R. G. Canti. The growth and development of psittacosis virus in tissue cultures. This *Journal*, 1935, xl, 231-241.

1937

R. G. Canti, J. O. W. Bland and Dorothy S. Russell. Tissue culture of gliomata. *Proc. Assoc. Res. Nerv. Ment. Dis.* (1935), 1937, xvi, 1-24.

1937-38

Microcinematography in biological research. *St Bart. Hosp. J.*, 1937-38, xlv, 253-254.

1938

J. O. W. Bland and Dorothy S. Russell. Histological types of meningiomata and a comparison of their behaviour in tissue culture with that of certain normal human tissues. This *Journal*, 1938, xlvii, 291-309.

C. F. Robinow and J. O. W. Bland. Application of the Feulgen method to the study of viruses. *Nature*, 1938, cxlii, 720 721.

1939

J. O. W. Bland and C. F. Robinow. The inclusion bodies of vaccinia and their relationship to the elementary bodies studied in cultures of the rabbit's cornea This *Journal*, 1939, xlviii, 381-403

Growth of human meningiomata in culture compared with that of certain human tissues *Arch. exp Zellforsch* , 1939, xx, 369 371.

1944

Spontaneous folliculosis of the conjunctiva in grivet and vervet monkeys (*Lasiopyga griseoviridis* syn *Cercopithecus œthiops* and *L. pygerythra* syn. *C pygerythra*) and the susceptibility of the grivet to trachoma virus. This *Journal*, 1944, lvi, 161-171.

Spontaneous folliculosis of the conjunctiva in baboons (*Papio hamadryas*). This *Journal*, 1944, lvi, 446.

1945

J. O. W. Bland and R. P. Wilson. Bacteriological and clinical observations on the treatment of the acute ophthalmias of Egypt with sulphonamides and penicillin. *Brit. J. Ophthalmol* , 1945, xxix, 339 355.

The ætiology of trachoma. A critical review of present knowledge. *Brit. J. Ophthalmol* , 1945, xxix, 407-420.

Stuart McDonald, Jr.
(Born 5th June 1905.　Died 24th October 1946)

(Plate CXIV)

Stuart McDonald, Jr., the only son of Professor Stuart McDonald, formerly Professor of Pathology in the University of Durham College of Medicine, was educated at Fettes College, Edinburgh. From there he went to Cambridge (Gonville and Caius College), where he graduated B.A. in 1926, and completed the preclinical part of the medical course. He pursued his clinical studies at the Royal Infirmary, Edinburgh, and Royal Victoria Infirmary, Newcastle-upon-Tyne. In April 1930 he obtained the diplomas of L.R.C.P. Lond. and M.R.C.S. Eng., and for six months acted as *locum-tenens* biochemist to the Royal Victoria Infirmary, Newcastle. In September of that year he was appointed demonstrator in pathology in the University of Durham College of Medicine, and assistant pathologist to the Royal Victoria Infirmary, and in December he graduated M.A. and M.B., B.Chir. Cantab. For the degree of M.B. he submitted an Act (thesis) entitled " The Schilling index in acute infections ". He held the Newcastle appointments till April 1934, when he was elected senior lecturer in pathology in the University of Birmingham, and assistant pathologist to the General Hospital. In July 1934 he was appointed histologist to the Birmingham branch of the British Empire Cancer Campaign. During his tenure of these various posts he was actively engaged in teaching, research and routine investigations. As a teacher he was outstanding, possessing in an unusual degree the ability to stimulate the enthusiasm of his students. He took a personal interest in them, was sympathetic towards their difficulties, and unsparing in his efforts to help them. He published numerous papers on a variety of subjects and made many communications to scientific societies. In all of these he showed himself to be a master of graphic and lucid exposition and possessed of a wide knowledge of the literature of his subject. Perhaps his chief interests lay in the direction of morbid anatomy and hæmatology, but he also did much work on other subjects, specially in the field of cancer research. His association with the British Empire Cancer Campaign involved observations on the classification and grading of tumours in connection with a " follow-up " scheme and with radiation therapy, the experimental production of mouse sarcomata by various polycyclic hydrocarbons and the growth of explants in the chick chorio-allantois, an experimental investigation of the effects of atmospheric dust on the mouse lung, and studies of the correlation between the quantitative excretion of gonadotropic urinary hormones and the histology of testicular tumours. In March 1938 he graduated M.D. Cantab., with a thesis entitled " A study of dibenzanthracene-induced mouse

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sarcomata, with special reference to the factors influencing the development of engrafted tumours ", and in December he received the degree of Ph.D. Birm., on submission of his contributions to pathological literature.

McDonald was a man of fine character and unusually attractive personality with whom, for many years, as colleague and friend, the writer was privileged to work in close association. He was a skilled and accurate observer, his judgment in the interpretation of difficult cases was at all times sound, and his hospital pathological work was much appreciated by his clinical colleagues. He made friends readily and was liked and respected by all who knew him, while his wide knowledge of men and things made him a desirable asset in any company. He did not, to any great extent, take an active part in games but he was interested in sport of all kinds, more especially angling and shooting, in which he achieved considerable success, and some of his happiest and certainly most entertaining reminiscences were of angling holidays spent with his father in various countries.

Always interested in military training, McDonald took a commission in the R.A.M.C. (T.A.) in 1932, and was for a time in command of the Durham University S.T.C. In 1936 he was given the mobilisation appointment of deputy assistant director of pathology, Shorncliffe Area, but on the formation of the 14th British General Hospital (T.A.) in Birmingham in 1939 he was transferred to this unit as its specialist in pathology, with the rank of major. The hospital was mobilised in August 1939, and with it McDonald served as a pathologist in England, France and India till August 1943, when the excellence of his work led to his appointment as principal histo-pathologist to the newly-formed Central Laboratory for the India Command at Poona. A year later he was transferred to the General Headquarters of the India Command at Delhi, as assistant director of pathology (research), with the rank of lieutenant-colonel. During his period of service in England and France he was engaged on investigations on the optimum fluid intake for patients on sulphonamide therapy and on sulphapyridine anuria. Whilst in India, in addition to an immense amount of routine work, he was responsible, in his capacity as A.D.P. (Research), for the initiation and administration of research in clinical pathology. This involved many long journeys, mostly by air, and he supervised the activities of no less than eight research teams which made important investigations on many matters intimately concerned with the health of the Forces in tropical countries, including scrub typhus, anæmia, amœbic dysentery and other protozoal diseases, epidemic jaundice, penicillin therapy, mepacrine therapy, arsphenamine nephritis and leprosy. Notwithstanding these manifold activities he found time to produce two army manuals dealing with post-mortem methods and histological methods. Colonel H. F. Humphreys, who, as O.C. 14th General Hospital, was closely associated with him during his period of service, has told the writer that McDonald was mess

president during more than half his time with the unit and that his tact, personal charm and social activities made him a universal favourite, and did much to promote the happiness and efficiency of the hospital, often under trying conditions of climate and inadequate accommodation.

At the end of 1944 he returned to this country on a short liaison visit to the India and War Offices and the Medical Research Council, and during this time he was interviewed at a special meeting of the appointing committee of the Court of the University of St Andrews. As a result he was appointed professor of pathology in the University of St Andrews and pathologist and clinical pathologist to the Dundee Royal Infirmary in March 1945.

He was demobilised later in the year and took up duty in September, full of enthusiasm and plans for continuing his work in this new environment. Unfortunately, after serving for a few weeks only, during which he had already inspired the trust and affection of his new colleagues and students, he had a severe breakdown and was ordered a prolonged period of rest. Latterly his condition seemed to be much improved and there was some prospect of his returning to duty, but he died suddenly and unexpectedly on 24th October 1946. His untimely death at the early age of 41 came as a great shock to his many friends, both in the Services and in civilian life, and it has robbed us of one of the most brilliant of our younger pathologists. He leaves a widow and two daughters and to them and to his father we extend our sincerest sympathy.

GEORGE HASWELL WILSON

BIBLIOGRAPHY

1931

Leucocyte counts in surgical prognosis. *Edin. Med. J.*, 1931, xxxviii, 657-688.

1931-32

C. GORDON IRWIN and STUART McDONALD, Jr. Two cases of localized phlegmonous enteritis. *Brit. J. Surg.*, 1931-32, xix, 362-373.

1932

A case of reticulum cell carcinoma of thymus. This *Journal*, 1932, xxxv, 1-9.
J. GILMOUR and STUART McDONALD, Jr. Aneurysm of abdominal aorta and thrombosis of superior mesenteric artery associated with a bullet wound of the lung. *Brit. Med. J.*, 1932, ii, 587-589.
Congenital syphilitic mesaortitis associated with acute yellow atrophy of the liver, with a note on the salvarsan-liver necrosis sequence. *Brit. J. Ven. Dis.*, 1932, viii, 263-291.

1933

A case of circumscribed lymphangeiomatous dilatation of the lingual papillæ. This *Journal*, 1933, xxxvi, 183-185.

A note on the pathology of Banti's disease with report of case. *Newcastle Med. J.*, 1933, xiii, 127-140.

1934

Syphilitic aortitis in young adults with special reference to a congenital ætiology. *Brit. J. Ven. Dis.*, 1934, x, 183-201.

Portal thrombosis secondary to duodenal ulcer. *Acta Chir. Scand.*, 1934, lxxiv, 405-416.

1936

Primary endothelioma of the pericardium. This *Journal*, 1936, xliii, 136-141.

1937

A. P. THOMSON, G. HASWELL WILSON and STUART MCDONALD, Jr. Three cases of unusual illness with eosinophilia, one with lesions in the liver resembling those of tularæmia. *Lancet*, 1937, ii, 9-13.

1938

STUART MCDONALD, Jr., and D. L. WOODHOUSE. The histology of transplants from a strain of 1 : 2 : 5 : 6-dibenzanthracene mouse sarcoma. This *Journal*, 1938, xlvii, 615-623.

Observations on chorionepithelioma testis with record of a case. *Amer. J. Cancer*, 1938, xxxiv, 1-14.

1939

STUART MCDONALD, Jr., and J. C. HEATHER. Neoplastic invasion of the pulmonary veins and left auricle. This *Journal*, 1939, xlviii, 533-543.

Some aspects of the cancer problem. *Birm. Med. Rev.*, 1939, xiv, 75-84.

1942

STUART MCDONALD, Jr., and D. L. WOODHOUSE. On the nature of mouse lung adenomata, with special reference to the effects of atmospheric dust on the incidence of these tumours. This *Journal*, 1942, liv, 1-12.

STUART MCDONALD, Jr., J. R. P. O'BRIEN and M. W. STANIER. Estimation of sulphonamide compounds in blood by the Lovibond comparator. *J. Roy. Army Med. Corps*, 1942, lxxix, 25-29.

1943

F. JACOBY, STUART MCDONALD, Jr., and D. L. WOODHOUSE. Growth of a dibenzanthracene-produced mouse sarcoma in the chorio-allantoic membrane of the chick. This *Journal*, 1943, lv, 409-417.

A case of benign neutropenia treated by sodium pentnucleotide. *Ind. Med. Gaz.*, 1943, lxxviii, 148-149.

1944

Bacterium enteritidis septicæmia. *Edin. Med. J.*, 1944, li, 320-326.

A method for the demonstration of Negri bodies. *Ind. J. Med. Res.*, 1944, xxxii, 205.

Notes on histological technique. Government of India Press, 1944.

Notes on post-mortem examination technique. Government of India Press, 1944.

1944-45

J. A. W. BINGHAM and STUART MCDONALD, Jr. A filarial nodule simulating a cyst of the external semilunar cartilage. *Brit. J. Surg.*, 1944-45, xxxii, 326-327.

1945

J. F. SHIRLAW, STUART MCDONALD, Jr., and W. HAYES. A case of canine *S. typhi-murium* infection, with notes on other Salmonella infections in animals. *Ind. J. Med. Res.*, 1945, xxxiii, 1-3.

Eric Biddle

(Born 7th June 1891: Died 21st August 1945)

ERIC BIDDLE, pathologist and transfusion officer to the East Suffolk and Ipswich Hospital, died on 21st August 1945 as the result of a motor accident on the previous evening. He took the Conjoint qualification in 1915 and won the M.C. and bar while serving with the R.A.M.C. Returning to Guy's, he took the M.B., B.S. London in 1922 and held appointments as house surgeon, out-patient clinical assistant and senior assistant bacteriologist. From the post of pathologist to the Devonshire Hospital, Buxton, he came in 1927 to the East Suffolk and Ipswich Hospital to become director of the new pathological laboratory from its opening in 1928.

Appreciations of Biddle's personal charm and success, coming from his clinical colleagues in Ipswich, were published in the *British Medical Journal* of 8th and 15th September and the *Lancet* of 15th September 1945. To the writer, whose acquaintance with Biddle dates from 1939, he gave ever-deepening friendship.

During the years 1939-45 Biddle was running his department very largely on a temporary and volunteer staff. He had earlier started a blood transfusion service, which he built up, organised and ran, dealing with 100-170 donors on alternate Sunday afternoons. Sunday mornings were largely devoted to his work as M.O. to the 9th Suffolk Home Guard Battalion. Medical-legal work made a heavy call on his time, and his skill and experience were highly valued by the local and central authorities concerned. On the evening prior to his fatal accident he had been discussing with the writer his hope of putting together his forensic experience in book form. Biddle kept himself in close touch with senior colleagues in London and Cambridge and, in particular, was a regular attender at the meetings of the British Medical Association (he was chairman of the East Suffolk division in 1939-40) and of the Association of the Clinical Pathologists. He was also treasurer of the Ipswich and District Clinical Association.

Outside his full professional life, he found time to devote to a music club which he had founded for the hospital's clinical and nursing staff. The club is now named after him. The Y.M.C.A. and St John's

Ambulance Brigade could also count on his help.   Photography appealed to him and his skill was always at the disposal of his medical colleagues.   He was an active member and one-time president of the Ipswich Photographic Society.

Biddle gave his friendship readily and his help unstintingly.   He was never easily perturbed and had a fund of anecdote and humour. One could but wonder that he was able to accomplish so much.   The success with which he helped so many, in ways so various, was clearly and well shown by the innumerable friends he had in Ipswich and for many miles around.

<div align="right">P. H. MARTIN</div>

Vladimir Glass
1906–1946

VLADIMIR GLASS was of Russian parentage and was born in Southern Russia in 1906.   His father's business involved a good deal of travel, but Vladimir always regarded Bakum as his home town, and his childhood's memories, which were very happy, were centred round his home there.   The revolution broke up his home and scattered the family, and Vladimir became a wanderer.   On one occasion, travelling down the Volga, the boat had to run the gauntlet of snipers of various marauding bands ; on another, he spent several days journeying tightly packed in a cattle truck and contracted typhus, and for over a year he suffered from severe muscular exhaustion.   Eventually he escaped to this country and, because of his difficulty with the language, was sent to a school in Suffolk for "backward" children.   Here his brilliant powers enabled him rapidly to overcome this initial handicap, and he entered University College, London, and subsequently the Medical School, where he had a most successful career.   In his undergraduate days he showed considerable skill as a draughtsman and did much modelling in clay, so that several of his friends at the Slade School thought that he was missing his vocation in studying medicine.   He qualified in 1931, and was house surgeon at U.C.H. and house physician at the Hospital for Nervous Diseases in Maida Vale.   He took the M.B., B.S. London in 1933.   After working at the School of Hygiene he obtained the diploma in bacteriology and, working with a grant from the Medical Research Council, he collaborated with Wooldridge and Knox in a paper on the variability in the activity of bacterial enzymes.   He then went to Liverpool as assistant bacteriologist in the City Laboratories under Hedley Wright, for whom he had a respect amounting to veneration, and a deep and warm affection. Under Wright's guidance Glass had full scope for his passion for investigation, and numerous papers, both in collaboration with others and from his own pen, testify to the breadth of his outlook and his

keen critical sense. In 1938, on Wright's advice, he took a post as assistant pathologist in the London County Council pathological service in order to widen his experience. Here he applied himself with enthusiasm to the practical work of clinical pathology and proved himself of the greatest help to his clinical colleagues in all matters of ward infection. He also became a skilled hæmatologist, and was continuously engaged in investigating problems with regard to the chemistry of bacterial action. Glass had a fine brain, a keen critical sense, and a wide knowledge of his subject, and was a most loyal colleague. His early and tragic death is a great loss to pathology.

G. W. GOODHART

BOOKS RECEIVED

Pathology of tumours

By R A WILLIS 1948 London Butterworth & Co Pp xxiii, 992 and 52, 500 text figs 63s

Here is the book which many of us " in the know " have been awaiting with high hopes and pleasurable anticipation " The spread of tumours in the human body ', published 14 years ago, gave us some idea of Professor Rupert Willis's capabilities as morbid anatomist and writer Now we have before us this much more ambitious work, a handsome volume of over 1000 pages, copiously illustrated, the garnered fruits of an intensive study of neoplasms and their literature over a period of some 20 years, during most of which the author held the appointment of pathologist to the Alfred Hospital, Melbourne The book has been completed during his two years' tenure of the Sir William H Collins chair of human and comparative pathology in the Royal College of Surgeons of England

The work aims at giving " a useful general outline of each topic " but is also intended as a personal record of the author's own observations and conclusions To this end many brief reports of personally studied cases, illustrative of the subjects under discussion are incorporated in the text, giving added point to the many figures derived from these sources All the figures are from personally studied material and most of them are new , nearly all are photomicrographs Wisely, the author has elected to illustrate, in the main, the less familiar appearances, and in particular the range of structure possible in the less common varieties of tumours Well known appearances of common tumours, such as are to be found in nearly every textbook of pathology, have often been omitted The result is a book which makes its appeal chiefly to the professional pathologist and cancer research worker, by whom, indeed, it will be found invaluable That it will be read with pleasure and profit by many other classes of reader there can be no doubt

The first 12 chapters (207 pages) are devoted to the general pathology of neoplasia and include chapters on classification and nomenclature, innocence and malignancy, experimental carcinogenesis, the statistical study of tumours, mode of origin, structure and growth, and metastasis The rest of the text (some 800 pages) deals systematically with the tumours of the various organs and tissues of the body.

There are many features of this work which deserve special mention, among them the copious and well chosen bibliographies at the end of each chapter The most important and useful references give the full title of the article and in addition have the author's name printed in clarendon capitals More often than not a brief appraisal is added A useful addition would have been a list of some of the best monographs on the whole subject of tumours, even where these already appear in the sectional bibliographies The very full and well arranged index, running to 52 pages, is another most commendable feature, particularly in a comprehensive work of this kind

Professor Willis always states quite clearly where he stands in (admittedly) controversial matters, preferring " to avoid non committal vagueness " and to state plainly his own present opinions, " even though

these may have to be modified in the light of future experience ". He is, for example, an out-and-out supporter of the squamous epithelial origin of simple pigmented moles and malignant melanomas, while failing to carry conviction to at least one reader of the correctness of his views ! We would only add that, after extensive sampling, we have come inevitably to the conclusion that this is a great work—sound, reliable, critical and compendious : it should be the prized possession of every pathologist who has to do with tumours. We congratulate Professor Willis on a notable achievement.

The parathyroid glands and skeleton in renal disease

By J. R. GILMOUR. 1947. London : Geoffrey Cumberlege, Oxford University Press. Pp. xiii and 157 ; 26 text figs. 18s.

This small monograph is a major contribution to the pathology of the parathyroids and a further step in the elucidation of the complex mechanism controlling calcium and phosphorus metabolism. Packed with the condensed records of a great amount of work, it needs to be read carefully and in conjunction with the author's two previous papers on the weight and on the histology of the normal parathyroids (Gilmour and Martin, this *Journal*, 1937, xliv, 431 ; Gilmour, this *Journal*, 1939, xlviii, 187). Enlargement of the parathyroids in association with renal disease has often been recorded but no precise study like this has appeared before. It records the results of a quantitative and histological investigation of the parathyroid tissue in 90 autopsies showing renal disease. The weight of the total parathyroid parenchyma, as distinct from the connective tissue and fat in the gland, is recorded in each case and compared with the normal figures obtained in the previous study (1937). Material from one or more bones was examined in 33 of the cases. Over 75 per cent. of the cases of primary subacute or chronic nephritis examined showed changes in the parathyroids and a less proportion of cases in which renal damage was secondary ; in the latter group long-standing ascending infection and obstruction were the most frequent causes of parathyroid changes.

The histology of several abnormal types of parathyroid is described ; two of these types are characteristic of renal disease, one (type II) being the usual type, while the other (type IV) was found in 5 cases with very greatly hypertrophied glands.

The broad conclusions of this work may be briefly summarised.

Any type of renal disease in which there is prolonged phosphorus retention with a rise in blood phosphorus will lead to changes in the parathyroids, at first qualitative, later quantitative. The qualitative changes are seen as an alteration in the histology indicating greater activity of the gland ; this is often followed by hypertrophy which sometimes reaches an extreme grade—in one case in this series over 60 times the mean normal weight. These changes involve all the parathyroids, thus differing from the neoplasia seen in von Recklinghausen's generalised osteitis fibrosa, which affects as a rule only one gland. When the parathyroids become sufficiently enlarged, bony changes in the form of osteitis fibrosa appear, the amount of osteitis fibrosa being proportional to the degree of parathyroid hypertrophy. In adults this is a pure osteitis fibrosa without osteomalacia ; in children " renal rickets " develops. Renal rickets is a combination of rickets and osteitis fibrosa, the osteitis fibrosa being the result of increased parathyroid activity.

The changes in the parathyroids represent a compensatory mechanism brought into action by the lowering of the blood calcium, which again is dependent on the increase in blood phosphorus. Ordinarily there is an

inverse relationship between the blood calcium and blood phosphorus, so that a high phosphorus is accompanied by a low calcium. Increased parathyroid activity accelerates osteoclasis and turns more calcium into the blood, thus tending to raise the blood calcium to normal or over in spite of a persistently high blood phosphorus. In cases of renal rickets the hyperactivity of the parathyroids, while promoting osteitis fibrosa, tends to heal the rickets. This is shown by the fact that in the seven cases here studied the amount of healing of the rickets was proportional to the degree of hypertrophy of the parathyroids. With extreme hypertrophy metastatic calcification, with or without calcinosis, is liable to develop.

The epidemiological significance of grouping and typing the hæmolytic streptococci

By JORGEN ERNST. 1942. Copenhagen ; Einar Munksgaard : London ; Geoffrey Cumberlege, Oxford University Press. Pp. 379 ; 4 text figs. 25s.

The first half of this monograph deals with the classification of hæmolytic streptococci. It opens with a review of the literature from the classification of streptococci proposed by Schottmüller in 1903 and traces the development of the various laboratory methods used for the differentiation of hæmolytic streptococci—hæmolysin production in fluid media (a curious omission is that of any reference to McLeod's work on this subject), fermentation of sugars and other biochemical methods, Lancefield's work on the antigenic analysis of hæmolytic streptococci and the precipitin method of grouping streptococci, and Griffith's type differentiation of group-A streptococci by the slide-agglutination technique. Reference is also made in a series of tables to biochemical and biological methods of classification used to differentiate strains in the other Lancefield groups from human and animal sources.

The writer's own studies on the methods used for the taking and transmission of swabs and cultures, the preparation of media, the grouping of hæmolytic streptococci and the typing of group-A strains are given in considerable detail. One observation which conflicts with the findings of most workers is the occurrence of a larger number of overlapping reactions in the group-precipitin test when the Fuller formamide method of extraction is used in comparison with the Lancefield acid-extraction method, but no explanation is offered.

Mention is made of two new provisional groups, L and M, described by Friedberg, and the author adds to these a new provisional group N, comprising 8 strains of hæmolytic streptococci isolated from milk. As groups L and M have already been described by Fry and Hare in 1939 and group N has been described by Shattock and Mattick in 1943, confusion is likely to arise and force is added to the necessity for co-ordination of streptococcal classification by the Streptococcal Committee of the International Society for Microbiology.

Of 1458 group-A strains examined 1170 were typed, and a surprisingly large number of strains belonging to groups B, C, G and "N" were also typed by the Griffith sera, other than types 7, 20 and 21 (group C) and 16 (group G). Fermentation types and sub-types of group-A organisms are described in relation to the agglutination types, and strains from a milk epidemic which could not be identified by the available agglutinating sera were identified by fermentation typing. The identity of the strains was later confirmed by cross-absorption tests with sera prepared from several strains.

The second half of the monograph deals with epidemiological studies of group-A infections. Studies were made of the distribution of serological

types causing scarlet fever, the frequency of hæmolytic streptococci in the throats of scarlet fever patients and the *occurrence* of cross infection and its relation to complications. Two milk-borne outbreaks due to group-A streptococci are described and an *account is given of two outbreaks of* scarlet fever in children's homes, and of an outbreak of inoculation infection due to type 11 involving 37 *children who were being immunised against* diphtheria. In general the results of the investigations confirm the findings of other workers in this country and in the U.S.A.

This work was published in Denmark in 1942 and the author has obviously not been able to take advantage of the more recent developments in streptococcal typing by the use of precipitin typing with type specific anti-" M " sera. This detracts somewhat from the value of the monograph as an up-to-date report on present knowledge. The work is well documented up to 1940 and provides for the uninitiated in one volume an admirable survey of the laboratory and field aspects of infections due to group-A hæmolytic streptococci. ·

Calcific disease of the aortic valve

By Howard T. Karsner and Simon Koletsky. 1947. Philadelphia, London, Montreal : J. B. Lippincott Company. Pp. viii and 111 ; 24 figs. on 12 plates. $5.

This is a report of an investigation into the nature of nodular calcifying sclerosis of the aortic valve. It is based on the authors' own study of 200 cases coming to post-mortem and it includes a historical review, a short chapter on clinical features and a list of about 85 references. At the beginning of the book is written " The evidence collected points strongly to the view that calcific disease of the aortic valve originates in inflammation and that the inflammation is usually, if not always, a manifestation of rheumatic fever ". Separating off their first group of 91 hearts showing gross evidence of rheumatic affection of the mitral valve and other areas apart from the aortic valve, the authors proceed to the demonstration of " microscopic stigmas " of rheumatism in 80 of the remainder. After whittling away at the 29 hearts left over, they agree to call them all rheumatic except four, only one of which stubbornly refused to yield any evidence at all of an inflammatory basis, another being regarded as " probably wholly negative ". Their analysis so impresses the authors that at the end of the book they write " When 196 of 200 hearts that were the seat of calcific disease of the aortic valve are shown to be of rheumatic origin, it might safely be assumed that all are in the same category ". The reviewer is not quite so deeply impressed. He would be inclined to dispute the admission of some of the evidence for rheumatic ætiology based on microscopic appearances alone. But whether one agrees with the conclusions or not, a painstaking study of an important condition like this is an achievement and the book will take its place in the literature of cardiac pathology. The reviewer is prepared to accept that the disease is a sequel of rheumatic infection in a substantial proportion of cases and that it is not due to a primary atherosclerosis, but bacterial endocarditis as an antecedent is hardly considered in this book and it is not improbable that recent experience of healing verrucose endocarditis under the influence of penicillin may re-awaken interest in this possibility.

Although a digression from the main thesis, it is interesting to note that while 190 patients were in hospital long enough to receive a clinical diagnosis, aortic stenosis was diagnosed in only 48 instances. The book is well produced but it is unfortunate that the plates all lie together in the

centro and are not referred to in the text. It is not an easy book to read, but it does contain an amount of information which future students of this disease may not ignore.

Bovine tuberculosis : including a contrast with human tuberculosis

By JOHN FRANCIS. 1947. London, New York and Toronto : Staples Press Ltd. Pp. 220 ; 30 figs. (6 in colour) on 17 plates and 6 text figs. 25s.

This book began as a review of bovine tuberculosis, but the scope was broadened to include a comparison with the disease in man. The double task has been well accomplished. The pathology of tuberculosis is fully described and admirably illustrated, and 400 references to the literature are given, not as a mere compilation, but as a source of facts and opinions which are critically examined to show the reasons for the author's judgment on the questions at issue.

Francis estimates that in Great Britain about 17-18 per cent. of cattle and 30-35 per cent. of dairy cows are tuberculous ; about 1 million cattle, representing 12 per cent. of the cattle population, are in tubercle-free herds. Estimates of the economic loss from this disease range from one to three million pounds per annum. The higher incidence of tuberculosis in dairy cows than in other cattle is attributed to their exposure to dust and droplet infection in the cowshed and to their higher average age, and not to the strain of milk production. In cattle, infection is nearly always aerogenic and, although extension of the disease may be slow, lung lesions seldom heal ; therefore nearly all tuberculin-positive cattle are infective for others.

The tuberculin test is discussed at length and reasons are given for adopting the single intradermal test. Although immunity can be produced in cattle by vaccination with B.C.G. or the vole acid-fast bacillus, eradication of the disease will depend on applying the tuberculin test and disposing of reactors. When the number of tuberculous cattle in a region is thus sufficiently reduced, "area eradication" can be accomplished by slaughtering the remaining reactors. If this process were gradually spread over the whole country, bovine tuberculosis could be practically eradicated from Great Britain as it has been from Finland, Guernsey, Norway and the United States of America. If progress in the creation of attested herds continues at the same rate as in 1938-39, the disease will have practically disappeared in 30 or 40 years. Meantime the 1000-2000 annual deaths from bovine tuberculosis in man can best be reduced by efficient pasteurisation of milk.

Studies on the formation of cellular substances during blood cell production

By BO THORELL. 1947. London : Henry Kimpton. Pp. 120 ; 14 figs. on 7 plates and 45 text-figs. 12s.

The application of new techniques to old problems is always exciting and often rewarding. Bo Thorell describes in this monograph the results obtained by investigating the development of hæmopoietic tissue with a combination of the method of microspectrographic analysis used by Caspersson and his colleagues and the special staining methods of Feulgen and Rossenbeck. The methods used for microspectrography are described in considerable detail. One is a photographic method combined with photometry, for use in the ultra-violet range, which is of particular value

for estimating the concentration of certain nucleic acids discussed later; the other is a photo-electrical method for working in the visible spectral range used for estimating hæmoglobin. The author claims that it is possible to determine amounts of the order of magnitude of 10^{-5} µg. within a single bone-marrow cell in the living state.

"Growth", he states, can be defined "as the new formation of the fundamental substances of the cell. As the latter consists mainly of protein substances the formation of protein can be regarded in general during cell growth as the quantitatively dominating process". The whole study is therefore based on analyses of the development and loss of certain substances closely associated with protein synthesis within the cell, namely ribose nucleic acid and ribodesose nucleic acid. In the first part of the monograph Thorell shows that in blood cells, as in other growing cells, the new formation of cellular proteins during hæmatopoiesis takes place at an early stage of development in the presence of high concentrations (>5 per cent.) of ribose polynucleotides in the cytoplasm and nucleolar apparatus. During blood-cell maturation the ribose polynucleotide-containing nucleolar mass and the concentration of polynucleotides decrease continuously, parallel with a declining activity of the cell. This decreasing growth activity of the individual blood cell during maturation is regulated by a definite part of the chromatin, which can be defined as the nucleolus-associated chromatin. The nucleolus therefore exerts a great influence upon the process of maturation.

In the second part Thorell describes observations which suggest to him that the endocellular synthesis of hæmoglobin does not start before the ribose polynucleotide metabolism (associated with the basic cellular protein substance) is finished and it then rises rapidly. Translated into morphological terms this means that he finds no significant quantities of hæmoglobin in cells older than the polychromatic erythroblast in normal marrow. In the marrow cells of pernicious anæmia, however, hæmoglobin is found in the presence of cytoplasmic ribose polynucleotides. In the marrow associated with hæmorrhagic anæmias the cells show a normal curve for nucleic acid but a deficiency in the development of hæmoglobin. He also describes disturbances of ribose nucleic acid metabolism in the characteristic cells from different types of leukæmia.

This monograph, though obviously of the greatest possible interest to hæmatologists, should be read by all workers interested in the living cell. It gives cytologists a new tool ; for instance, the process of normal maturation associated with loss of ribose polynucleotide in cells he believes to be reversible. In the case of the normal dentine cell of the growing tooth, lack of vitamin C causes a retrogression to an undifferentiated growing cell. Thus from the inert chromocentre of the mature dentine-cell nucleus new ribose polynucleotide containing nucleolar substance is developed, after which ribose polynucleotides appear in the cytoplasm. What happens in the dentine cell there is reason to believe from other facts advanced by Thorell can happen in other cells. In other words, apart from anything else, this new method may enable us to analyse at least in part how maturing agents like folic acid or destructive agents like radiations exert their effects. Thorell and his colleagues are primarily biologists rather than hæmatologists and clearly their results in the field of hæmatology will require to be checked and developed by experts in this particular field. Criticism of certain points in their technical procedures can be made, but this monograph, which is well illustrated with tables, graphs and photomicrographs, can certainly be recommended as a clear and stimulating record of experimental work in an already well-tilled field, using new methods which should be capable of wide application to biological problems.

Approaches to tumor chemotherapy

Edited by FOREST RAY MOULTON 1947 Washington, D C The American Association for the Advancement of Science Pp x and 442, 193 text figs $7 75

Research Conferences have been held in successive years (1944 1947) at Gibson Island, Maryland, under the auspices of the American Association for the Advancement of Science The volume under review contains the papers and discussions of the 1945 and 1946 Conferences relating to tumour chemotherapy, together with certain invited papers designed to complete the account of our present knowledge in this field

Following a historical introduction, the papers have been grouped so as to proceed from animal to human experimentation, in so far as the distinction is possible The papers as a whole describe and summarise almost all of present day methods in tumour chemotherapy

In the pithy historical introduction, relating to selected papers published since the opening of this century, Woglom touches briefly upon the main classes of treatments which have been advanced, e g administration of tumours or their extracts or autolysates, organotherapy, enzymes, metals, alterations in oxygen supply, fever treatment, cryotherapy, hormones and other chemical substances " If we have no cure to day, surely it is not from lack of trying " Sections on special methods, nutritional factors, bacterial products, nitrogen mustards and clinical aspects follow The section on nitrogen mustards represents a nearly complete account of all the work performed to date

On the whole the experimental results are more depressing than the clinical This is inevitably so, because the experimentalist aims at complete disappearance or cure of the test tumour, whereas the clinician is content with prolongation of life It has been proved beyond doubt that anti androgenic treatment of prostatic carcinoma in man and urethane treat ment of selected cases of leukæmia are life prolonging procedures The position of the nitrogen mustards is still *sub judice*

In these papers and discussions the view is often expressed that when a specific and reliable cure for cancer is eventually found, reports of success will begin to appear from all over the world A plea is added for the publication in short form of negative results, in order to save future workers from unnecessary labour and to guide them in planning their experiments

Illuminants and illumination for microscopical work

By F E J OCKENDEN 1947 London Williams & Norgate Pp 26, 7 figs on 3 plates and 9 text figs 2s 6d

In many ways this is just what the reviewer has been waiting for— plain mercantile information about the various light sources useful to the microscopist, of the kind that the electrical salesman would scarcely know, and pleasantly garnished with enough scientific data to tickle the laboratory palate This is a " Quekett Microscopical Club Monograph ", being a revised separate publication of an article which appeared in the *Journal of the Quekett Microscopical Club* series 4, vol ii, no 3, December 1946, it certainly now reads both easily and explicitly Even in these austerity times it might have been worth enlarging both on the types of lamps produced by the various makers and on the different illuminating systems, but even in its small compass it will be valuable to all who have to use photomicrography The modern fashion of writing *va* as symbol for *watts*, although sad to the pious Scot is one well worth adopting

A text-book of pathology

By E. T. Bell. Sixth edition. 1946. London: Henry Kimpton. Pp. 910 ; 500 text figs. and 4 colour plates. ' 50s.

The sixth edition of Bell's text-book has appeared after an interval of only two years since the fifth, a tribute to its undoubted merits. It has been considerably enlarged (910 instead of 862 pages), and the English price has been raised from 45s. to 50s. The American price is $7, the equivalent of 35s. at the current rate of exchange. It would be interesting to know the exact destination of this substantial addition to the price which the unfortunate British student will have to pay.

" The total number of illustrations has been increased from 448 to 500 by the addition of 78 new figures and the omission of 26 old ones ". The illustrations have always been one of the strongest features of this now well-established text-book, and particularly its photographs of clinical cases and morbid anatomical specimens and its well-chosen photomicrographs : the drawings are much less satisfactory. " A large part of the text . . . has been revised and much new material has been introduced. Among the topics discussed more extensively . . . are vitamin deficiencies and tropical diseases ". The very full sectional bibliographies, with a brief statement as to the content of each paper quoted, constitute another commendable feature. Very few new references have been added, however, which may perhaps be accounted for by the short interval which has elapsed since the last edition.

Bell's classification of tumours (pp. 296-298) remains a law unto itself. There is, admittedly, no wholly satisfactory classification of new growths, and certainly Bell's is not to be commended. The suffix " blastoma " is used without any reference to whether the tumour is simple or malignant—mature or anaplastic. " Angioblastoma ", " Endothelioma " and " Mesothelioma " are given as main groups, like " Connective tissue tumors " and " Epithelial tumors ". Other main groups are headed " Myoblastoma ", " Tumors of nervous tissue ", " Mixed tumors of salivary glands " and " Tumors of the carotid body ", while " Leukæmia " appears under the subheading " Malignant lymphoma " in the group of " Tumors of the blood-forming tissues ". Under the group heading of " Epithelial tumors " are four subheadings :—A. Papilloma, B. Adenoma, C. Carcinoma, and D. Special forms of epithelial tumors. It is to be hoped that some better classification will be evolved before the seventh edition makes it appearance.

That said, we would commend this manual to both teachers and students of pathology as one well worthy of their attention.

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